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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

WOLFFE et al.

Application No.: 09/844,508

Filed: April 27, 2001

For: TARGETED MODIFICATION OF CHROMATIN STRUCTURE

Examiner: R. Akhavan

Group Art Unit: 1636

Confirmation No.: 9058

BRIEF ON APPEAL

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TABLE OF CONTENTS

USSN 09/844,508
8325-0014
S14-US1

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3/10/05 Michelle Hodson
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BRIEF ON APPEAL

Mail Stop Appeal Brief
Commissioner for Patents
Alexandria, VA 22313

Sir:

INTRODUCTION

Appellants submit in triplicate their brief on appeal in accordance with 37 C.F.R. §1.192. All claims were finally rejected under 35 U.S.C. § 112, first paragraph, and provisionally rejected under the judicially-created doctrine of obviousness-type double patenting, in a Final Office Action mailed August 10, 2004. A Notice of Appeal was filed December 10, 2004,

making a Brief on Appeal due on or before February 10, 2005. Accompanying this Brief is a Petition for a one-month extension of time, extending the deadline to March 10, 2005. Accordingly, this Brief is timely filed. Appellants respectfully request that the decision of the Examiner be reversed.

I. REAL PARTIES IN INTEREST

Sangamo BioSciences, Inc., the assignee of record of the above-referenced patent application, is the real party in interest in this matter.

II. RELATED APPEALS AND INTERFERENCES

Appellants are not aware of any related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-72 are currently pending in the above-referenced case (hereinafter "the application"). The application was originally filed on April 27, 2001 with claims 1 to 72. Claims 7, 9, 14-16, 34-42, 71 and 72 were withdrawn pursuant to a Restriction Requirement mailed November 19, 2002, with the understanding that upon the allowance of linking claim 1, the restriction requirement will be withdrawn and the claims 7, 9, 14 and 16 (Groups I-VI) would be examined in this application. Examined claims 1-6, 8, 10-13, 17-33 and 43-70 were variously amended in papers filed April 11, 2003; December 2, 2003 and May 25, 2004. Following a telephone conference with Examiners Akhavan and Leffers on October 27, 2004, Applicants amended the claims in an Amendment After Final, filed November 8, 2004, to make explicit what was previously implicit. These amendments were not entered. Accordingly, claims 1-72 are pending as shown in Appendix A. Examined claims 1-6, 8, 10-13, 17-33 and 43-70 remain

rejected under 35 U.S.C. § 112 first paragraph, and provisionally rejected under the judicially-created doctrine of obviousness-type double patenting.

IV. STATUS OF THE AMENDMENTS

In response to the Examiner's Final Office Action mailed August 10, 2004 and following a telephonic interview on October 27, 2004, Appellants filed an Amendment after Final on November 8, 2004 in which independent claims 1 and 43 were amended to make explicit that the molecules used in the claimed methods altered chromatin structure such that access to cellular DNA by a second molecule was facilitated. An Advisory Action was mailed on December 3, 2004, indicating that amendments to the claims would not be entered and reiterating the rejections. Thus, all claims remained rejected for the reasons set forth in the Final Office Action.

V. SUMMARY OF THE CLAIMS

The claimed subject matter relates to methods of altering chromatin structure in a region of interest in cellular chromatin and to methods for modulating expression of a gene.

The methods are based on the fact that fusion molecules comprising a DNA binding domain and at least one subunit protein of a chromatin remodeling complex or functional fragment of the subunit protein are capable of altering the structure of chromosomal chromatin in a region of interest (page 5, lines 17-21). Because the DNA binding domain of these fusion molecules binds to a particular region of interest, chromatin structure is remodeled in a site-specific way, namely in the region(s) of interest only. This targeted modification of chromatin is useful in a number of ways. For example, by providing access to cellular DNA sequences at particular location(s), targeted chromatin modification allows gene expression to be modulated by a second molecule that modulates gene expression (page 5, lines 9-16).

In any of the claimed methods, the cellular chromatin that is altered may be present in a plant cell (page 5, lines 23-24) or an animal cell (*e.g.*, human cell) (page 5, lines 23-24).

Furthermore, the fusion molecule may be a fusion polypeptide (page 5, lines 21-22). In the examined group of claims, the DNA-binding domain of the fusion molecule may comprise a zinc finger DNA-binding domain (page 5, lines 27-28). Similarly, in the examined claims, the subunit protein or functional fragment thereof acts as an enzyme (page 6, lines 3-4).

As noted above, the claimed methods of altering chromatin structure can facilitate a number of processes including detection of a sequence of interest within the chromatin (page 51, lines 13-17) (*e.g.,* a single nucleotide polymorphism, page 57, lines 8-9), activation of a gene of interest (page 6, lines 14-20), repression of a gene of interest (page 6, lines 14-20), and/or recombination between an exogenous nucleic acid and cellular chromatin (page 6, lines 14-20). This is because generation of an accessible region in the cellular chromatin (page 10, lines 10-13) in turn can facilitate binding of an exogenous molecule (*e.g.,* polypeptides, nucleic acids, small molecule therapeutics, minor groove binders, major groove binders and intercalators, page 10, lines 13-18) to the now-accessible cellular chromatin (page 10, lines 10-13). Importantly, targeted chromatin modification, as claimed, is a separate and distinct process from the processes it facilitates.

In any of the methods described herein, the region of interest may comprise a gene (page 7, lines 1-2), for example a gene encoding a product selected from the group consisting of vascular endothelial growth factor, erythropoietin, androgen receptor, PPAR-γ2, p16, p53, pRb, dystrophin and e-cadherin (page 7, lines 2-7).

The methods of altering chromatin structure may further comprise the step of contacting the cellular chromatin with a second molecule (page 7, lines 8-10), for example a transcriptional regulatory protein (page 7, lines 12-13) or a fusion molecule such as a fusion polypeptide (page 7, lines 13-14). In certain embodiments, the second molecule comprises a zinc finger DNA-binding domain (page 7, lines 14-16). The second molecule may further comprise a transcriptional activation domain (page 7, lines 16-17), a transcriptional repression domain (page 7, lines 16-17) or a polypeptide sequence selected from the group consisting of a histone acetyl

transferase, a histone deacetylase, a functional fragment of a histone acetyl transferase, and a functional fragment of a histone deacetylase (page 7, lines 20-23).

Furthermore, the methods of altering chromatin structure may further comprise the step of contacting the cellular chromatin with a third molecule (page 7, line 30 to page 8, line 1), for example a transcriptional regulatory protein (page 8, lines 5-6) or a fusion molecule such as a fusion polypeptide (page 8, lines 6-9). In certain embodiments, the third molecule comprises a zinc finger DNA-binding domain (page 8, lines 7-8). The zinc finger DNA-binding domain of the third molecule may further comprise a transcriptional activation domain (page 8, lines 8-9) or a transcriptional repression domain (page 8, lines 8-9).

The subject matter of the examined claims also pertains to methods for modulating expression of a gene (page 8, lines 22-23). The methods comprise contacting chromosomal cellular chromatin with a first fusion molecule comprising a DNA-binding domain and at least one subunit protein of a chromatin remodeling complex or functional fragment (page 8, lines 22-29). The DNA-binding domain binds to a binding site in the gene (page 8, line 24). These methods further comprise contacting the cellular chromatin with a second molecule that binds to a target site in the gene and modulates expression of the gene (page 8, lines 27-29). The modulation may comprise activation of expression of the gene (page 6, lines 16-17) or repression of expression of the gene (page 6, lines 16-17).

In any of the methods of modulating gene expression, the first fusion molecule and/or the second molecule may comprise a zinc finger DNA-binding domain (page 8, lines 28-29 and page 9, lines 6-7). The second molecule may comprise a polypeptide (page 8, line 31), for example a zinc finger DNA-binding domain (page 9, lines 6-7), which may optionally further comprise an activation domain (page 9, lines 6-8) or a repression domain (page 9, lines 6-8). In any of the methods of modulating gene expression, the second molecule may be an exogenous or endogenous transcription factor (page 9, lines 2-4).

In any of the methods of modulating expression of a gene, a plurality of first fusion molecules (*e.g.*, zinc finger DNA-binding domain-containing molecules), may be contacted with

cellular chromatin, wherein each of the first fusion molecules binds to a distinct binding site (page 9, lines 9-11). Similarly, a plurality of second molecules (*e.g.*, zinc finger DNA-binding domain-containing molecules) can be contacted with cellular chromatin, wherein each of the second molecules binds to a distinct target site (page 9, lines 11-13).

Further, methods of modulating the expression of a plurality of genes are also provided (page 9, lines 18-19), for example by contacting cellular chromatin with a plurality of first fusion molecules (*e.g.*, zinc finger fusion polypeptides comprising a zinc finger DNA-binding domain, page 9, lines 22-23), wherein each of the first fusion molecules binds to a distinct binding site (page 9, lines 19-22). In addition to contacting cellular chromatin with a plurality of first fusion molecules as described above, the expression of a plurality of genes can be modulated by contacting cellular chromatin with a plurality of second molecules (*e.g.*, zinc finger fusion polypeptides comprising a zinc finger DNA-binding domain), wherein each of the second molecules binds to a distinct target site (page 9, lines 28-30). The first fusion molecule (*e.g.*, a zinc finger fusion polypeptide) may bind to a shared binding site in two or more of the plurality of genes (page 9, lines 23-25). Similarly, the second molecule (*e.g.*, a zinc finger fusion polypeptide) may binds to a shared target site in two or more of the plurality of genes (page 10, lines 2-4).

VI. ISSUES ON APPEAL

1. Whether amending the claims (i) to recite that the fusion molecule that alters chromatin structure does not in and of itself modulate gene expression or (ii) to recite that the alteration of chromatin structure by the first fusion molecule facilitates modulation of gene expression by a second molecule, violates the written description requirement of 35 U.S.C. § 112, first paragraph by adding new matter.

2. Whether the specification as filed enables examined claims 1-6, 8, 10-13, 17-33 and 43-70 throughout their scope under 35 U.S.C. § 112, first paragraph.

3. Whether the rejection of examined claims 1-6, 8, 10-13, 17-33 and 43-70 under the judicially created doctrine of obviousness-type double patenting in view of U.S. Serial No. 10/084,826 can be held until indication of allowable subject matter and then addressed in the reference application (of which the pending application is the parent).

VII. GROUPING OF CLAIMS

Claims 1-6, 8, 10-13, 17-33 and 43-70 are separately patentable, enabled and described by the application as filed. Therefore, these claims are divided into 57 separate groups:

(1) Claim 1: Independent claim 1 is drawn to a method for altering chromatin structure in a region of interest in chromosomal cellular chromatin, the method comprising the step of contacting the chromosomal cellular chromatin with a fusion molecule that binds to a binding site in the region of interest, wherein the fusion molecule comprises a DNA binding domain and at least one subunit protein of a chromatin remodeling complex or functional fragment of the subunit protein, wherein the contacting is conducted under conditions such that the structure of chromosomal chromatin is altered in the region of interest, and further wherein the fusion molecule does not regulate transcription

(2) Claim 2: Claim 2 is drawn to the method of claim 1 and further specifies that the cellular chromatin is present in a plant cell.

(3) Claim 3: Claim 3 is drawn to the method of claim 1 and further specifies that the cellular chromatin is present in an animal cell.

(4) Claim 4: Claim 4 is drawn to the method of claim 3 and further specifies that the animal cell is a human cell.

(5) Claim 5: Claim 5 is drawn to the method of claim 1 and further specifies that the fusion molecule is a fusion polypeptide.

(6) Claim 6: Claim 6 is drawn to the method of claim 1 and further specifies that the DNA-binding domain comprises a zinc finger DNA-binding domain.

(7) Claim 7: Claim 7 is drawn to the method of claim 1 and further specifies that the DNA-binding domain is a triplex-forming nucleic acid or a minor groove binder.

(8) Claim 8: Claim 8 is drawn to the method of claim 1 and further specifies that the subunit protein or functional fragment thereof acts as an enzyme.

(9) Claim 10: Claim 10 is drawn to the method of claim 1 and further specifies that the alteration of chromatin structure facilitates detection of a sequence of interest within the chromatin.

(10) Claim 11: Claim 11 is drawn to the method of claim 10 and further specifies that the sequence of interest comprises a single nucleotide polymorphism.

(11) Claim 12: Claim 12 is drawn to the method of claim 1 and further specifies that the alteration of chromatin structure facilitates activation of a gene of interest.

(12) Claim 13: Claim 13 is drawn to the method of claim 1 and further specifies that the alteration of chromatin structure facilitates repression of a gene of interest.

(13) Claim 17: Claim 17 is drawn to the method of claim 1 and further specifies that the region of interest comprises a gene.

(14) Claim 18: Claim 18 is drawn to the method of claim 17 and further specifies that the gene encodes a product selected from the group consisting of vascular endothelial growth factor, erythropoietin, androgen receptor, PPAR-γ2, p16, p53, pRb, dystrophin and e-cadherin.

(15) Claim 19: Claim 19 is drawn to the method of claim 1 and further specifies that the step of contacting the cellular chromatin with a second molecule.

(16) Claim 20: Claim 20 is drawn to the method of claim 19 and further specifies that the second molecule is a transcriptional regulatory protein.

(17) Claim 21: Claim 21 is drawn to the method of claim 19 and further specifies that the second molecule is a fusion molecule.

(18) Claim 22: Claim 22 is drawn to the method of claim 21 and further specifies that the second molecule is a fusion polypeptide.

(19) Claim 23: Claim 23 is drawn to the method of claim 21 and further specifies that the second molecule comprises a zinc finger DNA-binding domain.

(20) Claim 24: Claim 24 is drawn to the method of claim 23 and further specifies that the second molecule further comprises a transcriptional activation domain.

(21) Claim 25: Claim 25 is drawn to the method of claim 23 and further specifies that the second molecule further comprises a transcriptional repression domain.

(22) Claim 26: Claim 23 is drawn to the method of claim 23 and further specifies that the second molecule further comprises a polypeptide sequence selected from the group consisting of a histone acetyl transferase, a histone deacetylase, a functional fragment of a histone acetyl transferase, and a functional fragment of a histone deacetylase.

(23) Claim 27: Claim 27 is drawn to the method of claim 19 and further specifies that the further comprising the step of contacting the cellular chromatin with a third molecule.

(24) Claim 28: Claim 28 is drawn to the method of claim 27 and further specifies that the third molecule is a transcriptional regulatory protein.

(25) Claim 29: Claim 29 is drawn to the method of claim 27 and further specifies that the third molecule is a fusion molecule.

(26) Claim 30: Claim 30 is drawn to the method of claim 29 and further specifies that the third molecule is a fusion polypeptide.

(27) Claim 31: Claim 31 is drawn to the method of claim 29 and further specifies that the third molecule comprises a zinc finger DNA-binding domain.

(28) Claim 32: Claim 32 is drawn to the method of claim 31 and further specifies that the third molecule further comprises a transcriptional activation domain.

(29) Claim 33: Claim 33 is drawn to the method of claim 31 and further specifies that the

third molecule further comprises a transcriptional repression domain.

(30) Claim 43: Independent Claim 43 is drawn to the method of modulating gene expression. In particular, the method comprises contacting chromosomal cellular chromatin with a first fusion molecule that binds to a binding site in the gene (and in the chromosomal cellular chromatin). The first fusion molecule comprises a DNA-binding domain and at least one subunit protein of a chromatin remodeling complex or functional fragment of the subunit protein. The contacting is conducted under conditions such that the structure of chromosomal chromatin is altered in a region of interest. Subsequently, the cellular chromatin is further contacting with a second molecule that binds to a target site in the gene and modulates expression of the gene.

(31) Claim 44: Claim 44 is drawn to the method of claim 43 and further specifies that the modulation comprises activation of expression of the gene.

(32) Claim 45: Claim 45 is drawn to the method of claim 43 and further specifies that the modulation comprises repression of expression of the gene.

(33) Claim 46: Claim 46 is drawn to the method of claim 43 and further specifies that the DNA-binding domain of the first fusion molecule comprises a zinc finger DNA-binding domain.

(34) Claim 47: Claim 47 is drawn to the method of claim 43 and further specifies that the second molecule is a polypeptide.

(35) Claim 48: Claim 48 is drawn to the method of claim 47 and further specifies that the second molecule comprises a zinc finger DNA-binding domain.

(36) Claim 49: Claim 49 is drawn to the method of claim 48 and further specifies that the

second molecule further comprises an activation domain.

(37) Claim 50: Claim 50 is drawn to the method of claim 48 and further specifies that the second molecule further comprises a repression domain.

(38) Claim 51: Claim 51 is drawn to the method of claim 43 and further specifies that the second molecule is a transcription factor.

(39) Claim 52: Claim 52 is drawn to the method of claim 51 and further specifies that the transcription factor is an exogenous molecule.

(40) Claim 53: Claim 53 is drawn to the method of claim 51 and further specifies that the transcription factor is an endogenous molecule.

(41) Claim 54: Claim 54 is drawn to the method of claim 43 and further specifies that the first fusion molecule and the second molecule each comprise a zinc finger DNA-binding domain

(42) Claim 55: Claim 55 is drawn to the method of claim 43 and further specifies that the a plurality of first fusion molecules is contacted with cellular chromatin, wherein each of the first fusion molecules binds to a distinct binding site.

(43) Claim 56: Claim 56 is drawn to the method of claim 43 and further specifies that a plurality of second molecules is contacted with cellular chromatin, wherein each of the second molecules binds to a distinct target site.

(44) Claim 57: Claim 57 is drawn to the method of claim 55 and further specifies that at least one of the first fusion molecules comprises a zinc finger DNA-binding domain.

(45) Claim 58: Claim 58 is drawn to the method of claim 56 and further specifies that at least one of the second molecules comprises a zinc finger DNA-binding domain.

(46) Claim 59: Claim 59 is drawn to the method of claim 43 and further specifies that the expression of a plurality of genes is modulated.

(47) Claim 60: Claim 60 is drawn to the method of claim 59 and further specifies that a plurality of first fusion molecules is contacted with cellular chromatin, wherein each of the first fusion molecules binds to a distinct binding site.

(48) Claim 61: Claim 61 is drawn to the method of claim 59 and further specifies that at least one of the first fusion molecules is a zinc finger fusion polypeptide.

(49) Claim 62: Claim 61 is drawn to the method of claim 59 and further specifies that a plurality of second molecules is contacted with cellular chromatin, wherein each of the second molecules binds to a distinct binding site.

(50) Claim 63: Claim 63 is drawn to the method of claim 62 and further specifies that at least one of the second molecules is a zinc finger fusion polypeptide.

(51) Claim 64: Claim 64 is drawn to the method of claim 59 and further specifies that the first fusion molecule binds to a shared binding site in two or more of the plurality of genes.

(52) Claim 65: Claim 65 is drawn to the method of claim 64 and further specifies that the first fusion molecule is a zinc finger fusion polypeptide.

(53) Claim 66: Claim 66 is drawn to the method of claim 59 and further specifies that the second molecule binds to a shared target site in two or more of the plurality of genes.

(54) Claim 67: Claim 67 is drawn to the method of claim 66 and further specifies that the second molecule is a zinc finger fusion polypeptide.

(55) Claim 68: Claim 68 is drawn to the method of claim 1 and further specifies that the alteration of chromatin structure results in the generation of an accessible region in the cellular

chromatin.

(56) Claim 69: Claim 69 is drawn to the method of claim 68 and further specifies that generation of the accessible region facilitates binding of an exogenous molecule.

(57) Claim 70: Claim 70 is drawn to the method of claim 69 and further specifies that the exogenous molecule is selected from the group consisting of polypeptides, nucleic acids, small molecule therapeutics, minor groove binders, major groove binders and intercalators.

VIII. ARGUMENTS

1. Amendments Made During Prosecution Did Not Add "New Matter"

The first issue remaining in this case is whether the specification teaches methods as claimed, in which a fusion molecule made up of a DNA binding domain and a component of a chromatin remodeling complex is used to remodel chromatin **without** itself modulating expression of the gene. In other words, the issue is whether the specification as filed adequately describes the fact that the function of remodeling chromatin is separable from the function of modulating gene expression.

For the reasons of record, and those reiterated herein, Appellants submit that chromatin remodeling activity and transcriptional regulation are clearly described as separate functions in the specification as filed, and therefore that both the entered amendments of May 25, 2004 and the unentered amendments after final (November 8, 2004) included matter that was described in the specification as filed.

(a) No New Matter Was Added by Amendments filed May 25, 2004 and November 8, 2004

In their first attempt to address the issue of separate remodeling and regulatory functions, Appellants filed an Amendment on May 25, 2004, in which independent claims 1 and 43 were amended to recite that "the fusion molecule [comprising a DNA-binding domain and a subunit protein of a chromatin remodeling complex or functional fragment] does not regulate

transcription." In this Amendment, Appellants cited page 5, lines 9-12 of the specification, which states the following:

> These compositions are useful for facilitating processes that depend upon the access of cellular DNA sequences to DNA-binding molecules, for example, transcription, replication recombination, repair and integration.

In the Final Office Action, however, The Examiner determined that this Amendment somehow added new matter and that the so-called "negative limitation" was not supported by the specification as a whole (Final Office Action, page 3).

Following an interview with Examiners Akhavan and Leffers, Appellants filed an Amendment After Final on November 8, 2004. In a sincere attempt to advance prosecution, Appellants attempted to replace the objectionable phrase "fusion molecule does not regulate transcription" with a positive recitation that remodeling by the fusion protein facilitates access to cellular chromatin by a second molecule. Appellants cited the following teachings of the specification, all of which clearly indicate that the chromatin remodeling-DNA binding protein fusions used in the **claimed** methods do not regulate transcription themselves, but rather facilitate access to cellular chromatin, which may in turn allow binding of a molecule involved in transcriptional regulation (see, page 4, line 28 to page 5, line 6; page 5, lines 9-16; page 6, lines 14-15; page 12, lines 5-10; page 12, lines 18-19; page 13, lines 6-9, emphasis added):

> Despite this knowledge of the effects of chromatin remodeling on gene expression *in vitro* and *in vivo*, methods for directed manipulation of chromatin structure are not available. Accordingly, for situations in which a regulatory molecule is prevented, by chromatin structure, from interacting with its target site, methods for targeted modification of chromatin structure are needed. Such methods would be useful, for example, to **facilitate binding of regulatory molecules to cellular chromatin** and/or to **facilitate access of DNA-binding molecules to cellular DNA sequences**. This, in turn, would facilitate regulation of gene expression, either positively or negatively, by endogenous and exogenous molecules, and provide additional methods for binding these molecules to binding sites within regions of interest in cellular chromatin.
>
> Disclosed herein are compositions and methods useful for targeted modification of chromatin. These compositions and methods are useful for **facilitating**

> **processes that depend upon access of cellular DNA sequences to DNA-binding molecules**, for example, transcription, replication, recombination, repair and integration. In one embodiment, targeted modification of chromatin facilitates regulation of gene expression by endogenous or exogenous molecules, **by providing access to cellular DNA sequences**. Modification is any change in chromatin structure, compared to the normal state of the chromatin in the cell in which it resides.
>
> Modification of chromatin structure will **facilitate many processes that require access to cellular DNA**.
>
> Disclosed herein are compositions and methods useful for modifying chromatin structure in a predetermined region of interest in cellular chromatin. Modification of chromatin structure **facilitates many processes involving nucleotide sequence-specific interaction of molecules with cellular chromatin**. In certain embodiments, modification of chromatin structure is a prerequisite for binding of a regulatory molecule to its target site in cellular chromatin. Such binding can be useful in the regulation of an endogenous cellular gene by one or more endogenous and/or exogenous molecules.
>
> **Alterations in chromatin structure in the vicinity of the promoter**, mediated by the recruited remodeling complex, **facilitate subsequent interactions** that result in transcriptional activation or repression.
>
> Chromatin remodeling ensues in the vicinity of the target site, which renders the region of binding (*e.g.*, a gene promoter) susceptible to the action of endogenous regulatory factors, and/or to the regulatory activities of exogenous molecules.

Thus, in their Response After Final, Appellants clearly set forth that the specification teaches that, in certain embodiments, the fusion molecule used to remodel chromatin does not itself modulate gene expression (but, rather, facilitates the modulation of gene expression by making cellular chromatin accessible to the molecules that do modulate gene expression). Despite this clear support, the amendments after final were not entered. In fact, the after final amendments were alleged to change the nature and scope of the claimed subject matter. (Advisory Action, p. 2). Accordingly, the claims on appeal include language alleged to introduce new matter, as do the unentered after final amendments.

(b) The Amendments Filed May 25, 2004 are Fully Described by the as-filed Specification

In response to Appellants' arguments that the phrase "the fusion molecule does not regulate transcription" did not introduce new matter, the Examiner alleged that "the negative limitation ... distinctly directs the invention to particular fusion proteins that can never, whether indirectly or directly, regulate transcription. Such a genus is not supported by the disclosure or the particular portions of the disclosure Applicant cites." (Advisory Action, page 2).

There is no supporting basis for the Office's assertions that the genus of fusion molecules that do not themselves regulate transcription is not described in the specification and that the amendment distinctly directs the invention to fusion proteins that never indirectly regulate transcription.

With regard to the first assertion, the specification is replete with disclosures teaching that, in certain embodiments, the fusion proteins used to alter chromatin structure do not themselves have additional functions, but, rather, facilitate other functions by different molecules. *See,* passages cited above. It is thus clear that the claimed "genus" of fusion molecules that remodel chromatin but do not themselves regulation transcription is clearly described in the specification as filed. Moreover, it is axiomatic that an Applicant is always entitled to amend her claims so as to be directed to certain preferred embodiments.

There is also no basis in fact for the assertion that the amendment "distinctly directs" the claims to methods in which the claimed fusion proteins never indirectly regulate transcription. As used by the Examiner, the term "indirectly" seems to cover embodiments in which the alteration of chromatin structure by one molecule facilitates access to (and regulation of) a gene by another molecule. As noted above, such embodiments are plainly culled out in the specification and Appellants properly amended the claims to be directed to such embodiments. Thus, the specification amply describes the genus of methods in which the first fusion protein does not itself regulate transcription, but facilitates transcriptional activation or repression by different molecules. Accordingly, no new matter was added as a result of this amendment and the claims as on appeal (Appendix A) are described by the as-filed specification.

(c) The Amendments Filed After Final Did Not Change the Scope of the Invention

With regard to the (unentered) Amendments After Final, it was alleged that (Advisory Action, page 2):

> ...the amendments change the scope of the invention from one of a method of altering chromatin structure with a fusion protein that does not regulate transcription to a method where the chromatin structure is altered so as to facilitate access to cellular DNA, which necessarily includes access to regulate transcription (whether intended or not). Therefore, the claims as considered in the preceding action were directed to a distinct invention as compared to the claims as currently amended.

Appellants note that the alleged change in scope simply makes explicit what is positively recited in examined claim 43 (and claims dependent therefrom). Indeed, examined claim 43 is directed to a method of modulating gene expression by altering chromatin structure with a first fusion molecule and modulating gene expression with a second molecule. This "invention" has already been deemed by the Office to be part of the group of claims directed to methods of altering chromatin structure and has been examined with these claims. There is, therefore, nothing about the amendments that changes the scope of the claimed subject matter.

In sum, the amendments to the claims made by Appellants during prosecution are fully supported and described by the specification as filed. They do not introduce new matter and are not directed to a distinct invention. By refusing to enter these amendments, which were made in a sincere effort to clarify the claims and advance prosecution, the Office has unduly prolonged prosecution.

2. The Specification Fully Enables the Claims Throughout Their Scope

Claims 1-6, 8, 10-13, 17-33 and 43-70 have been repeatedly rejected under 35 U.S.C. §112, first paragraph as allegedly not enabled by the specification as filed. In particular, it was acknowledged that the specification enables *in vitro* methods but was alleged not enable *in vivo* methods.

In their Response After Final, Appellants cited the following passage on page 51, lines 18-22 of the specification:

> Targeted modification of chromatin structure, as disclosed herein, can be used in processes such as, for example, therapeutic regulation of disease-related genes, engineering of cells for manufacture of protein pharmaceuticals, pharmaceutical discovery (including target discovery, target validation and engineering of cells for high throughput screening methods) and plant agriculture.

In addition, further evidence of *in vivo* enablement was attached to that Response, namely articles establishing that the functional domain of a fusion protein (comprising a DNA binding domain and the functional domain) performs the same function *in vivo* as it does *in vitro*. *See*, Appendices B, C and D, attached hereto. Given that fusions of DNA binding domains and transcriptional activation domains function *in vivo* to modulate transcription, a skilled artisan would expect that fusions of DNA binding domains and chromatin remodeling proteins would also function *in vivo* to remodel chromatin, as set forth in the pending application.

However, in the Advisory Action, the arguments and references were deemed unpersuasive on the grounds that the functional domains of the references were not chromatin remodeling proteins or subunits thereof. (Advisory Action, page 4). For the reasons of record and reiterated below, Appellants submit that a *prima facie* case of non-enablement has not been established and the specification fully enables the claims throughout their scope.

(a) A *Prima Facie* Case of Non-Enablement Has Not Been Established

As set forth in the seminal case of *In re Marzocchi,* 439 F.2d, 220, 223, 169 USPQ 367, 369 (CCPA 1971), a patent application is presumptively enabled when filed:

> [a]s a matter of Patent Office practice ... a specification .. must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

Moreover,

> it is incumbent upon the Patent Office, whenever a rejection on [grounds of enablement] is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure.

439 F.2d at 224, 169 USPQ at 369-370. Indeed, as pointed in the Patent Office's own Training Manual on Enablement (1993, citing *In re Wright,* 999 F.2d 1557, 1561-1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993), "the case law makes clear that properly reasoned and supported statements explaining any failure to comply with section 112 are a requirement to support a rejection."

In the case on appeal, the Examiner has not properly set forth why the teachings of the specification, particularly the passage reproduced above from page 51, do not enable the practice of the claimed methods *in vivo*. The Office has not provided any reason to doubt that the claimed methods are operative *in vivo,* especially given the evidence of record establishing that zinc finger-functional domain fusion proteins perform *in vivo* exactly as predicted from their *in vitro* properties.

It is not sufficient for the Examiner to assert, without supporting evidence, that the cited articles are not relevant because "an activation domain and a remodeling domain are two biologically distinct structures with disparate functions." (Advisory Action, page 4). In the absence of evidence supporting and describing the alleged distinctness of the two domains, the rejection cannot be maintained.[1]

In addition, the Examiner has mischaracterized the nature of Appellants' arguments, stating that "[i]f one were to accept Applicant's contention, then a fusion protein consisting of a

[1] Indeed, the Office has consistently held that chromatin remodeling and transcription modulation are inseparable processes. *See* section 1 above regarding 35 U.S.C. § 112, first paragraph (written description) and the Office's previous rejection under 35 U.S.C. § 103(a) as set forth *e.g.*, in the Office Action dated February 25, 2004

DNA domain and *any* functional domain would be enabled for *in vivo* application by Applicant's disclosure/cited art."

First and foremost, Appellants have not made any statements regarding *any* general fusion protein. Rather, Appellants have noted that the specification as filed teaches *in vivo* applications and no evidence has been presented that speaks against this contention.

Moreover, Appellants have not argued that *any* functional domain would work *in vivo*. Rather, Appellants presented evidence that analogous (not equivalent) molecules that were known at the time of filing to function *in vitro* also function in the same way *in vivo*. The logic of Appellants' argument is simple: the specification is acknowledged by the Office to enable *in vitro* methods using fusions of DNA-binding domains and chromatin remodeling proteins; the specification teaches one of skill in the art how to practice the methods *in vivo*; the art teaches that fusions of DNA-binding domains and transcriptional activation domains work in the same way *in vitro* and *in vivo* and that the methods do not need to be altered as between *in vitro* and *in vivo* applications. Accordingly, one of skill in the art would conclude that following the teachings of the specification for the admittedly enabled *in vitro* methods would also work, without modification, in *in vivo* methods. Appellants' argument is not as broad as painted in the Advisory Action and, indeed, such an argument is unnecessary given that a *prima facie* showing of lack of enablement has not been made.

(b) The Specification Fully Enables *In vivo* Methods

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *Ex parte Forman*, 230 USPQ 546 (BPAI 1986). *See, also,* M.P.E.P. § 2164.01 which states the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art, citing *United States v. Telectronics Inc.*, 8 USPQ2d 1217 (Fed. Cir. 1988), *cert. denied,* 490 U.S. 1046 (1989)).

In the pending case, there is no argument that the claimed methods are fully enabled for *in vitro* applications. Furthermore, the specification teaches that the *in vitro* methods can be practiced *in vivo* without modification. The evidence of record (Appendices B, C and D) establishes that there is no difference in function between *in vitro* and *in vivo* methods, for analogous DNA-binding domain-activation domain fusions.

Thus, Appellants have provided ample factual evidence demonstrating that the specification enables the pending claims throughout their scope and the rejection should be withdrawn.

3. Appellants Did Not Traverse the Obviousness-Type Double Patenting

The Advisory Action also improperly states that Appellants did not "provide any arguments as to why the [obviousness-type double patenting] rejection is improper. .... This is not a proper ground for traversal." (Advisory Action, page 4).

Appellants did not traverse the obviousness-type double patenting rejection of claims 1-6, 8, 10-13, 17-33 and 43-70 over claims 1-33 and 44-71 of co-pending Application No. 10/084,826. Instead, Appellants noted that the pending application is the parent of the reference application[2] and that once claims were allowed in one application, the provisional rejection could be properly addressed in the other, namely the subsequently filed reference application. Given that the parent application is much farther along in prosecution, Appellants simply requested that the provisional ODP rejection be held and addressed in the subsequently-filed application. *See* also MPEP 804.I.B.

4. Additional Arguments Regarding Separately Grouped Claims

Each one of the preceding arguments is applicable to all of the separately grouped claims, *i.e.*, to each claim individually. For the sake of brevity, the arguments have been set out primarily as to independent claims 1 and 43. Claims 2-6, 8, 10-13, 17-33 and 44-70 (which have

[2] Appellants also note that a double patenting rejection in the reference application (USSN 10/084,826) over the present application has been made (*See* Office Action dated January 21, 2005 in USSN 10/084,826).

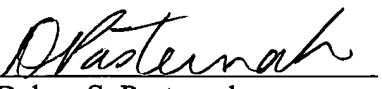
been rejected for the same reasons) contain all the elements of claims 1 or 43 and are, therefore, enabled and described by the specification as filed for the reasons discussed in detail above.

CONCLUSION

For the reasons stated above, Appellants respectfully submit that the pending claims are clearly described in the specification and fully enabled across their entire scope. Accordingly, Appellants request that the rejections of the claims on appeal be reversed, and that the application be remanded to the Examiner so that the appealed claims can proceed to allowance.

Respectfully submitted,

Date: March 10, 2005

By: 

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APPENDIX A:

LIST OF CLAIMS ON APPEAL

CLAIMS ON APPEAL

1. (previously presented): A method for altering chromatin structure in a region of interest in chromosomal cellular chromatin, the method comprising the step of contacting the chromosomal cellular chromatin with a fusion molecule that binds to a binding site in the region of interest, wherein the fusion molecule comprises a DNA binding domain and at least one subunit protein of a chromatin remodeling complex or functional fragment of the subunit protein, wherein the contacting is conducted under conditions such that the structure of chromosomal chromatin is altered in the region of interest, and further wherein the fusion molecule does not regulate transcription.

2. (original): The method of claim 1, wherein the cellular chromatin is present in a plant cell.

3. (original): The method of claim 1, wherein the cellular chromatin is present in an animal cell.

4. (original): The method of claim 3, wherein the cell is a human cell.

5. (previously presented): The method of claim 1, wherein the fusion molecule is a fusion polypeptide.

6. (original): The method of claim 1, wherein the DNA-binding domain comprises a zinc finger DNA-binding domain.

7. (withdrawn): The method of claim 1, wherein the DNA-binding domain is a triplex-forming nucleic acid or a minor groove binder.

8. (previously presented): The method of claim 1, wherein the subunit protein or functional fragment thereof acts as an enzyme.

9. (withdrawn): The method of claim 1, wherein the subunit protein or functional fragment thereof is non-enzymatic.

10. (previously presented): The method of claim 1, wherein the alteration of chromatin structure facilitates detection of a sequence of interest within said chromatin.

11. (original): The method of claim 10, wherein the sequence of interest comprises a single nucleotide polymorphism.

12. (previously presented): The method of claim 1, wherein the alteration of chromatin structure facilitates activation of a gene of interest.

13. (previously presented): The method of claim 1, wherein the alteration of chromatin structure facilitates repression of a gene of interest.

14. (withdrawn) The method of claim 1, wherein chromatin modification facilitates recombination between an exogenous nucleic acid and cellular chromatin.

15. (withdrawn) The method of claim 5, wherein the method further comprises the step of contacting a cell with a polynucleotide encoding the fusion polypeptide, wherein the fusion polypeptide is expressed in the cell.

16. (withdrawn): The method of claim 1, further comprising the step of identifying an accessible region in the cellular chromatin, wherein the fusion molecule binds to a target site in the accessible region.

17. (original): The method of claim 1, wherein the region of interest comprises a gene.

18. (original): The method of claim 17, wherein the gene encodes a product selected from the group consisting of vascular endothelial growth factor, erythropoietin, androgen receptor, PPAR-γ2, p16, p53, pRb, dystrophin and e-cadherin.

19. (original): The method of claim 1, further comprising the step of contacting the cellular chromatin with a second molecule.

20. (original): The method of claim 19, wherein the second molecule is a transcriptional regulatory protein.

21. (original): The method of claim 19, wherein the second molecule is a fusion molecule.

22. (original): The method of claim 21, wherein the second molecule is a fusion polypeptide.

23. (original): The method of claim 21, wherein the second molecule comprises a zinc finger DNA-binding domain.

24. (original): The method of claim 23, wherein the second molecule further comprises a transcriptional activation domain.

25. (original): The method of claim 23, wherein the second molecule further comprises a transcriptional repression domain.

26. (original): The method of claim 23, wherein the second molecule further comprises a polypeptide sequence selected from the group consisting of a histone acetyl transferase, a histone deacetylase, a functional fragment of a histone acetyl transferase, and a functional fragment of a histone deacetylase.

27. (original): The method of claim 19, further comprising the step of contacting the cellular chromatin with a third molecule.

28. (original): The method of claim 27, wherein the third molecule is a transcriptional regulatory protein.

29. (original): The method of claim 27,wherein the third molecule is a fusion molecule.

30. (original): The method of claim 29,wherein the third molecule is a fusion polypeptide.

31. (original): The method of claim 29, wherein the third molecule comprises a zinc finger DNA-binding domain.

32. (original): The method of claim 31, wherein the third molecule further comprises a transcriptional activation domain.

33. (original): The method of claim 31, wherein the third molecule further comprises a transcriptional repression domain.

34. (withdrawn): A fusion polypeptide comprising:

a) a DNA binding domain; and

b) a component of a chromatin remodeling complex or a functional fragment thereof.

35. (withdrawn): The polypeptide of claim 34, wherein the DNA-binding domain is a zinc finger DNA binding domain.

36. (withdrawn): The polypeptide of claim 34, wherein the DNA binding domain binds to a target site in a gene encoding a product selected from the group consisting of vascular endothelial growth factor, erythropoietin, androgen receptor, PPAR-γ2, p16, p53, pRb, dystrophin and e-cadherin.

37. (withdrawn): The polypeptide of claim 34, wherein the component of a chromatin remodeling complex or functional fragment thereof is an enzymatic component.

38. (withdrawn): The polypeptide of claim 34, wherein the component of a chromatin remodeling complex or functional fragment thereof is a non-enzymatic component.

39. (withdrawn): The polypeptide of claim 37, wherein the enzymatic component of a chromatin remodeling complex or functional fragment thereof is selected from the group consisting of a SWI/SNF complex family member, an Mi-2 complex family member, an ISWI complex family member, a BRM family member, a BRG/BAF complex family member, a Mot-1 complex family member, a Chd-1 family member, a Chd-2 family member, a Chd-3 family member, a Chd-4 family member, a histone acetyl transferase and a histone deacetylase.

40. (withdrawn): A polynucleotide encoding the fusion polypeptide of claim 34.

41. (withdrawn): A cell comprising the fusion polypeptide of claim 34.

42. (withdrawn): A cell comprising the polynucleotide of claim 40.

43. (previously presented): A method for modulating expression of a gene, the method comprising the steps of:

a) contacting chromosomal cellular chromatin with a first fusion molecule that binds to a binding site in the chromosomal cellular chromatin, wherein the binding site is in the gene and wherein the first fusion molecule comprises a DNA-binding domain and at least one subunit protein of a chromatin remodeling complex or functional fragment of the subunit protein, wherein the contacting is conducted under conditions such that the structure of chromosomal chromatin is altered in the region of interest and further wherein the fusion molecule; and

b) further contacting the cellular chromatin with a second molecule that binds to a target site in the gene and modulates expression of the gene.

44. (original): The method of claim 43, wherein modulation comprises activation of expression of the gene.

45. (original): The method of claim 43, wherein modulation comprises repression of expression of the gene.

46. (original): The method of claim 43 wherein the DNA-binding domain of the first fusion molecule comprises a zinc finger DNA-binding domain.

47. (original): The method of claim 43 wherein the second molecule is a polypeptide.

48. (original): The method of claim 47 wherein the second molecule comprises a zinc finger DNA-binding domain.

49. (original): The method of claim 48, wherein the second molecule further comprises an activation domain.

50. (original): The method of claim 48, wherein the second molecule further comprises a repression domain.

51. (original): The method of claim 43 wherein the second molecule is a transcription factor.

52. (original): The method of claim 51 wherein the transcription factor is an exogenous molecule.

53. (original): The method of claim 51 wherein the transcription factor is an endogenous molecule.

54. (original): The method of claim 43 wherein the first fusion molecule and the second molecule each comprise a zinc finger DNA-binding domain.

55. (original): The method of claim 43 wherein a plurality of first fusion molecules is contacted with cellular chromatin, wherein each of the first fusion molecules binds to a distinct binding site.

56. (original): The method of claim 43, wherein a plurality of second molecules is contacted with cellular chromatin, wherein each of the second molecules binds to a distinct target site.

57. (original): The method of claim 55 wherein at least one of the first fusion molecules comprises a zinc finger DNA-binding domain.

58. (original): The method of claim 56 wherein at least one of the second molecules comprises a zinc finger DNA-binding domain.

59. (original): The method of claim 43 wherein the expression of a plurality of genes is modulated.

60. (original): The method of claim 59 wherein a plurality of first fusion molecules is contacted with cellular chromatin, wherein each of the first fusion molecules binds to a distinct binding site.

61. (original): The method of claim 60 wherein at least one of the first fusion molecules is a zinc finger fusion polypeptide.

62. (original): The method of claim 59, wherein a plurality of second molecules is contacted with cellular chromatin, wherein each of the second molecules binds to a distinct binding site.

63. (original): The method of claim 62 wherein at least one of the second molecules is a zinc finger fusion polypeptide.

64. (previously presented): The method of claim 59 wherein the first fusion molecule binds to a shared binding site in two or more of the plurality of genes.

65. (original): The method of claim 64 wherein the first fusion molecule is a zinc finger fusion polypeptide.

66. (previously presented): The method of claim 59 wherein the second molecule binds to a shared target site in two or more of the plurality of genes.

67. (original): The method of claim 66 wherein the second molecule is a zinc finger fusion polypeptide.

68. (previously presented): The method of claim 1, wherein the alteration of chromatin structure results in the generation of an accessible region in the cellular chromatin.

69. (original): The method of claim 68, wherein generation of the accessible region facilitates binding of an exogenous molecule.

70. (original): The method of claim 69, wherein the exogenous molecule is selected from the group consisting of polypeptides, nucleic acids, small molecule therapeutics, minor groove binders, major groove binders and intercalators.

71. (withdrawn): A method for producing a fusion polypeptide, wherein the fusion polypeptide comprises a zinc finger DNA binding domain and a component of a

chromatin remodeling complex or a functional fragment thereof, the method comprising the step of expressing the polynucleotide of claim 40 in a suitable host cell.

72. (withdrawn): A method for binding an exogenous molecule to a binding site, wherein the binding site is located within a region of interest in cellular chromatin, wherein the method comprises:

(a) contacting cellular chromatin with a fusion molecule that binds to a binding site in the region of interest, wherein the fusion molecule comprises a DNA binding domain and a component of a chromatin remodeling complex or functional fragment thereof, thereby modifying cellular chromatin within the region of interest; and

(b) introducing the exogenous molecule into the cell;

whereby the exogenous molecule binds to the binding site.

APPENDIX B

Induction of angiogenesis in a mouse model using engineered transcription factors

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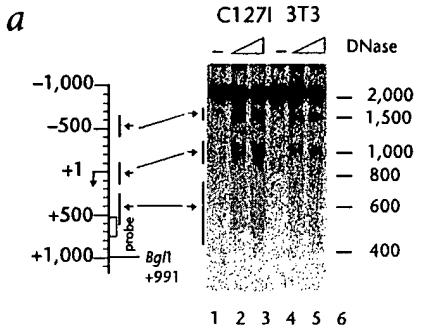
The relationship between the structure of zinc-finger protein (ZFP) transcription factors and DNA sequence binding specificity has been extensively studied[1]. Advances in this field have made it possible to design ZFPs *de novo* that will bind to specific targeted DNA sequences[2–10]. It has been proposed that such designed ZFPs may eventually be useful in gene therapy[6,7,10]. A principal advantage of this approach is that activation of an endogenous gene ensures expression of the natural array of splice variants[2]. Preliminary studies in tissue culture have validated the feasibility of this approach[2–4]. The studies reported here were intended to test whether engineered transcription factors are effective in a whole-organism model. ZFPs were designed to regulate the endogenous gene encoding vascular endothelial growth factor-A (*Vegfa*). Expression of these new ZFPs *in vivo* led to induced expression of the protein VEGF-A, stimulation of angiogenesis and acceleration of experimental wound healing. In addition, the neovasculature resulting from ZFP-induced expression of *Vegfa* was not hyperpermeable as was that produced by expression of murine $Vegfa_{164}$ cDNA. These data establish, for the first time, that specifically designed transcription factors can regulate an endogenous gene *in vivo* and evoke a potentially therapeutic biophysiologic effect.

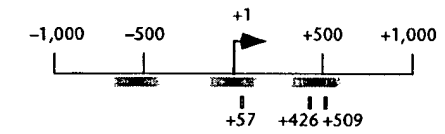
A central component of our strategy for designing ZFP transcriptional regulators was the preferential targeting of DNase I–accessible regions within the locus of interest. Such regions are more accessible to transcription factor–sized macromolecules than surrounding DNA, and often comprise binding sites for natural transcriptional regulators. We have found that designing ZFPs to preferentially target such regions yields both a higher success rate and a greater potency of response[2]. Accordingly, we mapped DNase I–accessible regions in the mouse *Vegfa* locus and observed three regions of enhanced accessibility centered approximately at bases –550, +1 and +400 (relative to the transcription start site) (Fig. 1*a*). We saw similar patterns of accessibility at the *Vegfa* locus in a variety of cell types from human and rat[2].

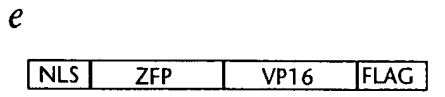
Choosing target sites within the '+1' and '+400' accessible regions (Fig. 1*b*), we designed ZFPs that could recognize these sequences with high affinity (Fig. 1*c*). To do this we linked together fingers of known triplet preference to yield either three- or six-finger ZFPs with the desired sequence specificities. Two of these designs, designated mVZ+426 and mVZ+509 (according to their target location relative to the transcription start site), contain three fingers and target sequences of nine base pairs conserved in human and mouse. The transcriptional activation properties of these proteins for the corresponding human gene, *VEGFA*, have been described[2] (referred to as, respectively, VZ+434 and VZ+42/+530 in that study). ZFP mVZ+57 contains six fingers and targets a sequence of 18 base pairs present only in the mouse. Gel-shift assays confirmed high-affinity binding of these specific ZFPs to their targeted *Vegfa* regulatory sequences (Fig. 1*d*). We constructed cytomegalovirus promoter (CMV)–driven expression plasmids encoding ZFP-based *Vegfa*-activating transcription factors by fusing sequences encoding the finger domains with the herpes simplex virus VP16 transactivation domain, a nuclear translocation signal and a FLAG epitope (Fig. 1*e*).

To test the ability of these ZFPs to activate *Vegfa* expression *in vitro*, we stably introduced ZFP expression cassettes into C127I cells by retroviral transduction. ZFP expression in these cells resulted in transcriptional upregulation of the endogenous *Vegfa* locus and secretion of VEGF-A (Fig. 1*f,g*). It has previously been shown that ZFP activation of *Vegfa* leads to production of the naturally occurring splice variants in their normal stoichiometry[2]. The intensity of *Vegfa* activation varied depending on the promoter region targeted.

The configuration of the chromatin encompassing a particular gene may vary among cell types and in response to the environment of the cell[11]. Given that transcription factors generally require a 'permissive' chromatin configuration for DNA binding and that the chromatin configuration *in vivo* might vary considerably from that existing *in vitro*, it was not known whether these artificial ZFP-based transcription factors could activate *Vegfa* and induce angiogenesis *in vivo* (in this context referring to events occurring within a living animal). To examine this, we inserted the mVZ+509, mVZ+57 and mVZ+426 expression cassettes into recombinant adenovirus vectors. Injection of these adeno-ZFP vectors into the quadriceps muscle of CD-1 mice resulted in substantially greater VEGF-A expression than did contralateral quadriceps injection with adenovirus carrying the gene encoding green fluorescent protein (adeno-*GFP*) (Fig. 2*a–c*). To ensure that *Vegfa* activation was not influenced by the presence of the adenovirus, this experiment was repeated with injections of plasmid DNA encoding the various *Vegfa*-activating ZFPs. To ensure that a nonspecific effect of ZFP expression or binding was not contributing to the *Vegfa* activation, a plasmid encoding the





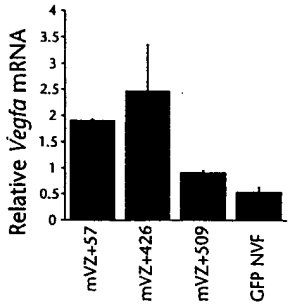


c

ZFP Name	Target Sequence 5'- 3'	Subsites 5'- 3'	Finger designs -1123456	
mVZ+57	TGAGCGGCGGCAGCGGAGc	TGAg	QSGHLTK	F6
		GCGg	RSDELSR	F5
		GCGg	RSDELTR	F4
		GCAg	QSGSLTR	F3
		GCGg	RSDELQR	F2
		GAGc	RSDNLAR	F1
mVZ+426	GGGGGTGACc	GGGg	RSDHLSR	F3
		GGTg	TSGHLVR	F2
		GACc	DRSNLTR	F1
mVZ+509	GCTGGGGGCg	GCTg	QSSDLTR	F3
		GGGg	RSDHLTR	F2
		GGCg	DRSHLTR	F1

d

ZFP	Target	Gel shift		Apparent K_d (nM)
mVZ+57	TGAGCGGCGGCAGCGGAGc		Bound Free	0.031
mVZ+426	GGGGGTGACc		Bound Free	<0.01
mVZ+509	GCTGGGGGCg		Bound Free	<0.01
SP1	GGGGCGGGGg		Bound Free	0.053



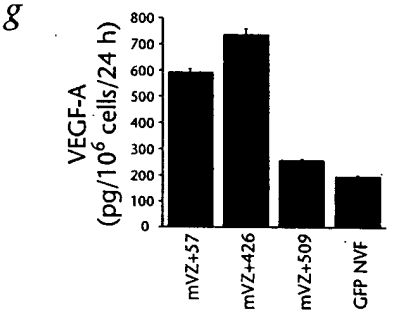


Fig. 1 Targeting, design and *in vitro* analysis of zinc-finger proteins designed for the mouse *Vegfa* locus. ***a***, Mapping of DNase I–accessible regions in the mouse *Vegfa* promoter region. Vertical bar, *Vegfa* promoter region; hooked arrow, transcription start site. Tick marks indicate units of 100 bp. The migration pattern for a set of DNA standard fragments is indicated on the right, with the size of each fragment given in base pairs. Arrows highlight the relationship of observed bands to location of accessible chromatin regions relative to the *Vegfa* transcription start site. DNase I concentrations (units/ml) in lanes 1–6 were as follows: 0, 32, 64, 0, 8 and 16. ***b***, Location of ZFP target sites. A schematic representation of the mouse *Vegfa* gene is provided, showing the location of the transcription initiation site (arrow) and the DNase I–accessible regions (gradient-filled rectangles) determined in these studies. ZFP target locations are indicated by dashes and the position of the upstream edge of each ZFP target is indicated by the number below it. Numbering is relative to the start site of transcription (+1). ***c***, ZFP target sequences and finger designs. ZFPs are named according to target site location and the suffix mVZ (for mouse *Vegfa* ZFP). Finger designs indicate the identity of amino acid residues at positions '–1' to '+6' of the alpha helix of each finger. ***d***, Gel-shift assays of target affinity. A 3-fold dilution series of each protein was tested for binding to its DNA target, with the highest concentration in lane 10 and the lowest concentration in lane 2. Lane 1 contains probe alone. Apparent K_d values, derived from the average of 3 such studies, are indicated at right. ***e***, Schematic representation of the ZFP transcriptional activators used in these studies. 'NLS','VP16' and 'FLAG' indicate the relative locations of, respectively, a nuclear localization signal, VP16 transactivation domain and FLAG tag. ***f***, ZFP-mediated activation of *Vegfa* transcription *in vitro*. Mouse-derived C1271 cells were stably transduced with retroviral vectors encoding each ZFP transcriptional activator or a control protein in which GFP was substituted for the ZFP DNA-binding domain. RT-PCR analysis is expressed as relative *Vegfa* mRNA. GAPDH served as a loading control. ***g***, As in (*f*), except that the amount of secreted VEGF-A protein was quantified by ELISA.

finger region of mVZ+57 without the VP16 transactivation domain was injected into the contralateral quadriceps as a control. These studies showed the expected *in vivo* activation of *Vegfa* by the designed ZFP and substantiated the notion that this activation was not a general response to the presence of the fingers but also required the VP16 transactivation domain (Fig. 2*d*).

To gain an initial sense of the magnitude and selectivity of *in vivo Vegfa* activation by our ZFP activators, we carried out quantitative RT-PCR analysis of RNA isolated from virus-treated quadriceps. In addition to *Vegfa*, these studies quantified relative expression of three non-target genes: *Glut1* (also known as *Slc2a1*), *Pgk1* and *Ldh1*, encoding glucose transporter-1, phosphoglycerate kinase and the A chain of lactate dehydrogenase-1, respectively. These genes are actively transcribed in skeletal muscle and would therefore be expected to present accessible chromatin regions capable of binding transcription factors. These genes are also induced by the hypoxic response, and were chosen to control for the possibility that our ZFPs were activating *Vegfa* by indirect effects acting through this pathway. Adenovirus encoding mVZ+57 was injected into the quadriceps muscle of CD-1 mice as above (n = 4 per group). Evaluation of gene expression three days later by real-time quantitative RT-PCR showed 266.7% (P = 0.005) more

Vegfa mRNA in mVZ+57-injected muscle as compared to adeno-*GFP*-injected contralateral controls (Fig. 2*e*). In contrast, mVZ+57 did not activate *Ldh1* or *Glut1* and activated *Pgk1* only slightly. As a positive control, an adenovirus encoding the hypoxia-inducible transcription factor HIF-1α was similarly tested and showed markedly greater expression of all these genes (Fig. 2*e*).

Further studies investigated effects of mVZ+509 and mVZ+426 on vascularization in the mouse ear angiogenesis model[12].

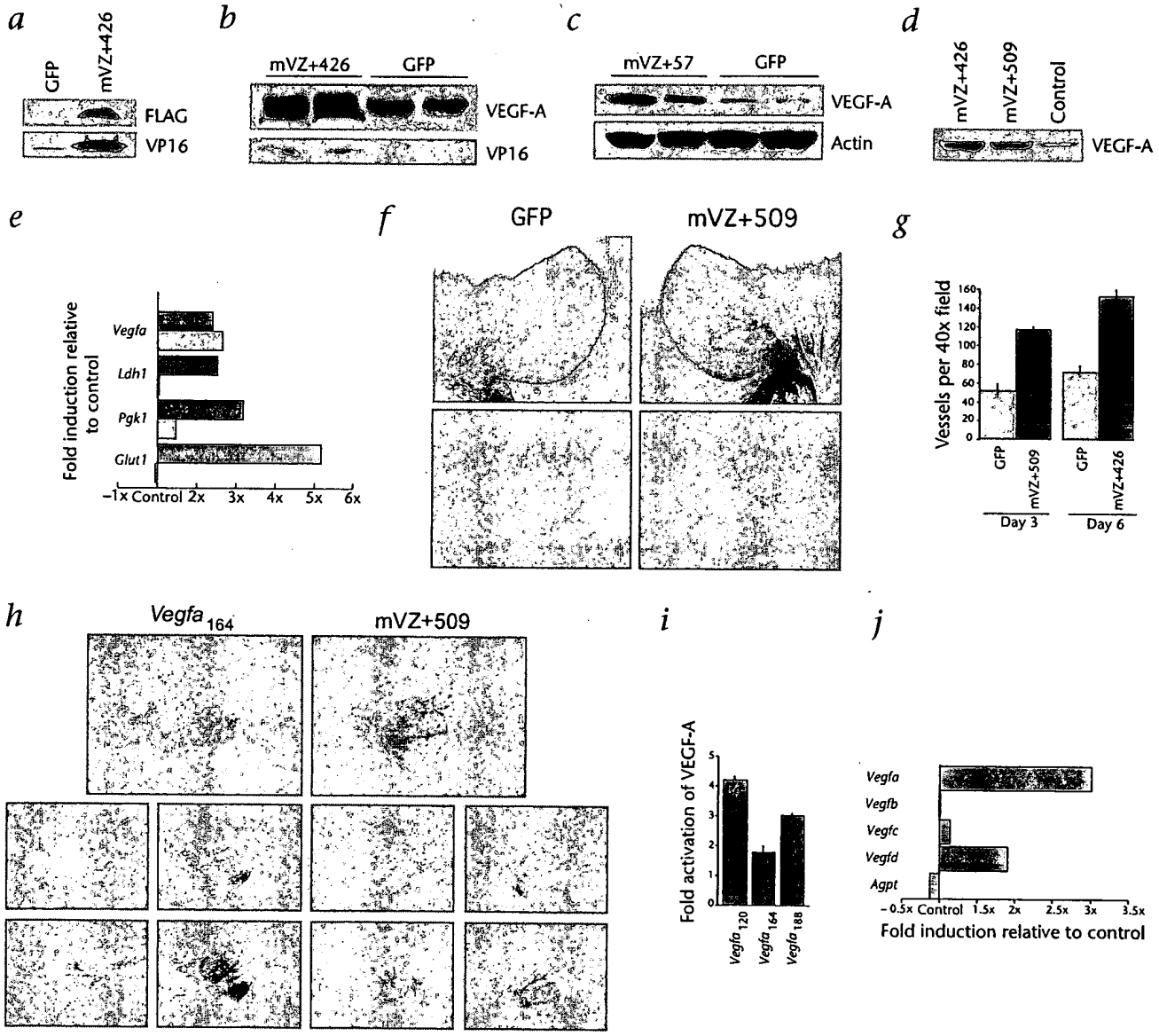


Fig. 2 ZFP-mediated *Vegfa* activation and induction of angiogenesis *in vivo*. ***a***, Western blotting of smooth muscle cells transduced with adenovirus encoding *Vegf*-ZFP; the presence of FLAG epitope and VP16 transactivation domain (TAD) demonstrates effective expression of the *Vegf*-ZFP. ***b,c***, Western blots showing VEGF-A in the quadriceps muscle of CD-1 mice 3 d after intramuscular injection with 10^8 PFU of adenovirus encoding either mVZ+426 or mVZ+57. VP16 TAD expression documents expression of the ZFP; actin expression documents equal loading. ***d***, Western blot of *Vegfa* expression in skeletal muscle 3 d after injection of plasmid DNA encoding either mVZ+426, mVZ+509 or a control plasmid encoding a *Vegfa*-targeting finger region without the VP16 TAD. ***e***, Comparison of profiles of gene activation induced by *Vegf*-ZFP mVZ+57 (light bars) and transcription factor HIF-1α (dark bars). Real-time RT-PCR shows that injection of adenovirus encoding human HIF-1α into the quadriceps muscle results in significant increases ($P \leq 0.01$) in the amounts of mRNAs for phosphoglycerate kinase (*Pgk1*), the glucose transporter (*Glut1*) and the lactate dehydrogenase A chain (*Ldha*) as well as activation of *Vegfa*. In contrast, mVZ+57 activates *Vegfa* without activation of *Glut1* or *Ldh1* and with only a minimal increase in *Pgk1* expression ($n = 4$ per group; all values compared to contralateral control quadriceps injected with adeno-*GFP*). ***f,g***, *Vegfa*-activating ZFP expression induces angiogenesis in the mouse ear. Subcutaneous injection of adenovirus encoding mVZ+509 results in visible neovascularization after 3 d relative to contralateral ears injected with adenovirus encoding GFP. Quantification by immunohistochemical vessel counts 3 d after mVZ+509 ($n = 5$) and 6 d after mVZ+426 ($n = 4$) corroborates the neovascularization. In *g*, $P < 0.001$ (left); $P = 0.001$ (right). ***h***, In similar experiments, angiogenesis stimulated by mVZ+509 (top and middle right) does not produce a hyperpermeable neovasculature as determined by Evans blue dye extravasation (bottom right). The neovasculature induced by $Vegfa_{164}$ adenovirus transduction (left) shows spontaneous hemorrhage and Evans blue extravasation. ***I***, Quantitative RT-PCR shows increases in mRNA of all 3 major *Vegfa* splice variants *in vivo* 3 d after VEGF-ZFP transduction. ***J***, RT-PCR shows increased total *Vegfa* mRNA, no substantial increases in *Agpt* (angiopoietin), *Vegfb* or *Vegfc* mRNAs, and a moderate increase in *Vegfd* mRNA in response to mVZ+509 ZFP transduction.

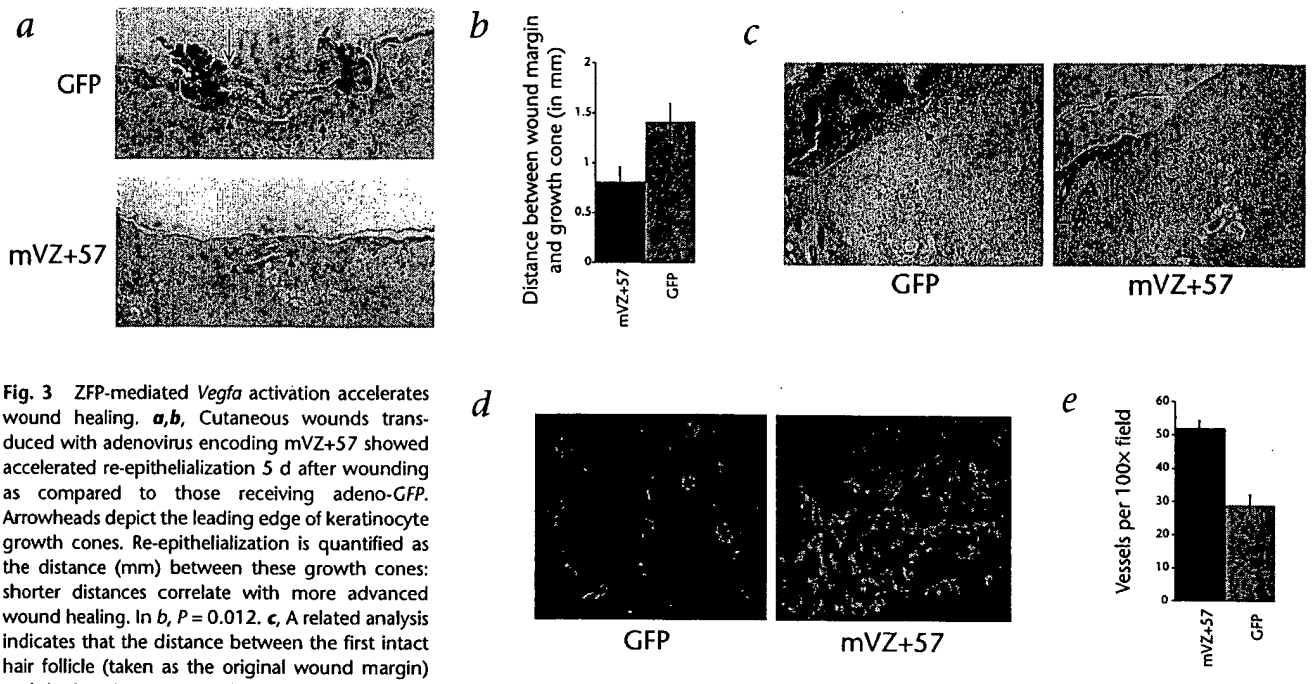


Fig. 3 ZFP-mediated *Vegfa* activation accelerates wound healing. ***a,b***, Cutaneous wounds transduced with adenovirus encoding mVZ+57 showed accelerated re-epithelialization 5 d after wounding as compared to those receiving adeno-*GFP*. Arrowheads depict the leading edge of keratinocyte growth cones. Re-epithelialization is quantified as the distance (mm) between these growth cones: shorter distances correlate with more advanced wound healing. In *b*, $P = 0.012$. ***c***, A related analysis indicates that the distance between the first intact hair follicle (taken as the original wound margin) and the keratinocyte growth cone is greater in the mVZ+57-treated wounds (arrows), indicating more rapid re-epithelialization. ***d–e***, Endothelial cell immunostaining of the same wounds shows an increase in vessel counts in the mVZ+57-transduced wounds at day 5 as compared to the GFP-transduced control wounds. In *e*, $P < 0.001$.

Subcutaneous injection of either construct in the external ear of CD-1 mice resulted in definitively greater vascularity in the ZFP-treated ears as compared to contralateral ears injected with adeno-*GFP* (Fig. 2*f*). These differences were readily apparent visually and were corroborated by immunohistochemical vessel counting: mVZ+509 111 ± 9.2 versus GFP 43.8 ± 7.7 per 100× field 3 days after transduction ($P = 0.005$, $n = 5$); mVZ+426 153.7 ± 10.0 versus GFP 74.7 ± 6.6 per 100× field 6 days after transduction ($P = 0.001$, $n = 4$) (Fig. 2*g*). VEGF-A is a potent vascular permeability factor that induces hemorrhage and extravasation of intravascular dye in the mouse ear model[12]. Notably, in contrast to the vasculature of ears similarly treated with an adenovirus carrying murine $Vegfa_{164}$ cDNA, the ZFP-induced neovasculature was not spontaneously hemorrhagic and was not permeable to infusion of Evans blue dye (Fig. 2*h*). Real-time quantitative RT-PCR of RNA isolated from treated and control ears showed ZFP-mediated increases in all three principal *Vegfa* splice variants but no increase in the mRNA for angiopoietin, a growth factor previously shown to be capable of promoting the growth of a more 'mature' non-leaky neovasculature (Fig.2*i,j*)[13]. Similarly, there was no apparent induction of *Vegfb* or *Vegfc*, although the amount of *Vegfd* (*Figf*) mRNA was 1.91 ± 0.27–fold greater than in controls.

To further examine the *in vivo* biological effects of these *Vegfa*-regulating ZFPs, we carried out adeno-mVZ+57 gene transfer into cutaneous wounds in a mouse model of wound healing[14]. Five days after wounding and gene transfer, wounds treated with adeno-mVZ+57 showed markedly accelerated re-epithelialization as quantified by the measured distance between keratinocyte growth cones: *Vegfa*-ZFP 0.8 ± 0.14 mm versus adeno-*GFP* 1.42 ± 0.15 mm ($P < 0.012$, $n = 6$ per group) (Fig. 3*a–c*). This was accompanied by a 63.4% greater vessel density in the ZFP-treated wounds as compared to GFP-treated control wounds in the same mouse ($P < 0.01$, $n = 5$) (Fig. 3*d,e*).

In these studies we demonstrate, for the first time, the feasibility of regulating genes and complex biological processes *in vivo* using engineered ZFP-based transcription factors. This represents a new paradigm in gene therapy that is predicated on the modulated expression of endogenous genes. There are considerable potential advantages to this approach, including the ability to regulate all the natural splice variants of an endogenous gene with a single deliverable effector molecule[2]. The *Vegfa* gene encodes a number of splice variants, and recent data suggest that these are not functionally equivalent or redundant[15]. In this study we show that the neovasculature resulting from activation of the endogenous *Vegfa* by engineered ZFPs is not hyperpermeable, as is that resulting from $Vegfa_{164}$ alone. It is possible that the ZFP-induced neovasculature is more physiologically mature and that this is due to the induced expression of the natural *Vegfa* splice variants. This hypothesis is supported by recent findings indicating that transcriptional upregulation of the endogenous *Vegfa* by the hypoxia-inducible factor HIF-1α also produces a non-hyperpermeable neovasculature[16]. It is also possible that another factor, not specifically targeted by the *Vegfa*-activating ZFPs, is upregulated in response to ZFP expression and contributes to the vascular phenotype. Expression of angiopoietin, an angiogenic factor capable of inducing a non-hyperpermeable neovasculature, is not induced in *Vegfa*-ZFP-transduced tissue. Expression of the VEGF family members VEGF-B and VEGF-C is also unresponsive to ZFP transduction, although that of VEGF-D was increased to a modest degree, albeit to a lesser extent than for the targeted VEGF-A. The reasons for the relatively small increase in VEGF-D are unclear. There are partial binding sites for mVZ+509 in the murine *Vegfd* promoter that may allow activation. It is also possible that these increases are secondary to the induced expression of VEGF-A. Despite this increase in VEGF-D expression, the total amount of *Vegfd* mRNA remained less than

approximately 1/12 that of *Vegfa* mRNA (data not shown). Whether this is enough to contribute substantively to the vascular phenotype is unknown.

Another advantage of ZFP-based gene regulation is that it facilitates the simultaneous regulation of multiple separate genes with a single therapeutic intervention. In especially favorable cases, this can be accomplished by designing a single ZFP that binds a DNA sequence common to all the desired genes. It can also be accomplished by combining expression cassettes encoding multiple ZFP constructs into a single gene-delivery vehicle. The relatively small size of these ZFP cassettes (~0.6–1.0 kb) facilitates the latter approach. Regulating multiple genes and their natural splice variants using a cDNA-based approach would be distinctly more difficult. This ability to regulate multiple endogenous genes through a single molecular intervention may prove very advantageous for the therapeutic modulation of complex biological processes that involve the coordinated expression of multiple genes. Currently, approaches to stimulate angiogenesis in ischemic hearts are based on the expression of single isoforms of angiogenic growth factors[17–19]. The ability to activate expression of multiple angiogenic genes and their splice variants with a single delivery vehicle, such as is facilitated by the ZFP-based approach, might provide a distinct advantage in the therapeutic regulation of angiogenesis. Establishing this, however, will require more extensive analysis in multiple *in vivo* models such as the ameroid model of coronary insufficiency and the hindlimb ischemia model[17,20]. Revascularization in these settings is complex, and whether ZFP-induced expression of *Vegfa* splice variants will be advantageous in such settings is currently unknown.

One crucial finding of the current study is the demonstration that synthetic transcription factors targeted to open regions of chromatin can specifically regulate targeted endogenous genes *in vivo*. We have previously shown that similar ZFP-based constructs can effect gene activation *in vitro*, but this was in the context of transformed and immortalized cell lines with a chromatin structure which may be different from that encountered in quiescent muscle cells *in vivo*. These data demonstrate, for the first time, the crucial next step: gene activation and induction of a therapeutic biophysiologic effect by an engineered ZFP transcription factor *in vivo*.

A crucial issue with respect to the use of designed transcription factors is specificity *in vivo*. As an initial step towards addressing this issue, our muscle studies included analyses of a set of non-target genes. The loci examined in these studies were chosen because they are transcribed in muscle (and therefore should contain accessible chromatin regions) and also because they are targets for the hypoxic response. We found little or no ZFP activation of these genes. In contrast, and as expected, these genes were activated by an adenovirus encoding HIF-1α, an endogenous transcription factor that is being developed as a potential pro-angiogenic gene-therapy agent[20].

ZFP-based transcription factors can be designed to activate, or when combined with a repression domain, to repress expression of nearly any endogenous gene. The inherent generality and versatility of this approach has notable implications for gene-based therapies predicated on the regulation of complex biological processes such as angiogenesis. These studies establish, for the first time, the feasibility and potential utility of this approach *in vivo*.

Methods

Mapping of DNase I–accessible regions of chromatin in the mouse *Vegfa* locus. Nuclei were isolated from C127I and NIH3T3 cells (ATCC, Manassas, Virginia) and treated with DNase I (Worthington, Lakewood, New Jersey) as previously described[3] except that digests were for 1.5 min at 22 °C (DNase I concentrations as indicated in Fig. 1). Genomic DNA isolation, restriction enzyme digestion and Southern-blot analysis were done as described[3] with enzymes and probes as indicated in Figure 1.

Synthesis of ZFP genes and ZFP binding studies. The assembly of genes encoding the three-finger ZFPs mVZ+426 and mVZ+509 has been described[2] (VZ+434 and VZ+42/+530, respectively, in that study). The gene encoding the six-finger protein mVZ+57 was assembled by joining genes encoding fingers 1–3 and 4–6 with a short DNA spacer encoding a flexible peptide linker (HQNKKGGSGDGKKKQHIC). Each resultant ZFP gene was cloned into the plasmid pMal (New England Biolabs, Beverly, Massachusetts) as a fusion with DNA encoding maltose-binding protein. Protein was isolated and binding studies done as described[2] with slightly modified buffer conditions (under these conditions SP1 shows a higher affinity than that seen in previous studies). ZFP concentrations were determined directly by measuring the DNA-binding activity of each ZFP preparation using conditions under which binding is essentially stoichiometric (concentrations of ZFP and target site $>50 \times K_d$).

Construction of ZFP-based transcription factors and vectors. To construct expression plasmids encoding *Vegfa*-activating ZFPs, the cassettes encoding each three- or six-finger ZFP were assembled by PCR with genes encoding the nuclear translocation signal from SV40 large T antigen, the herpes simplex virus VP16 transactivation domain spanning amino acids 413–490 and a FLAG peptide. They were subcloned into pcDNA3.1 (Clontech, Palo Alto, California) with expression under direction of the CMV promoter. Recombinant adenovirus vectors were constructed using the Ad-Easy system[21]. The MMLV-based retroviral vectors were derived from pLXSN, which contains a neomycin-resistance gene under the control of an internal SV40 promoter. The LXSN vectors were produced in the 293 Ampho-Pak cell line (Clontech).

***In vitro* studies.** To produce stable ZFP expressing cells, 293 Ampho-Pak and C127I cells (ATCC) were transduced with ZFP-encoding retrovirus and G418-resistant populations of cells were established 14 d later. Cells were plated (5×10^5 cells per well) and *Vegfa* expression evaluated by ELISA (R&D Systems, Palo Alto, California) on medium collected from wells 24 h after re-feeding. Adenovirus constructs were tested *in vitro* by transduction of rat aortic smooth muscle cells. At 24 h after transduction, cells were scraped and lysates used for western-blot analysis of VP16 and FLAG expression (see below).

***In vivo* skeletal muscle transduction.** Adenovirus encoding *Vegfa*-ZFP was injected intramuscularly into the quadriceps of CD-1 mice (10^8 PFU in 50 μl PBS) using a 27-gauge needle. Control vector was injected into the contralateral quadriceps. Plasmid DNA injection was done similarly using 50 μg of DNA. Mice were sacrificed after 3 d and muscle harvested for analysis of gene expression. Western blotting was done starting with muscle lysates using a monoclonal antibody against VEGF-A (Research Diagnostics, Flanders, New Jersey) and an ECL kit. Antibodies against FLAG (Sigma, St. Louis, Missouri) and the VP16 transactivation domain (Santa Cruz Biotechnology, Santa Cruz, California) were used to determine expression of the ZFPs. Antibody against actin was used to control for loading. For real-time quantitative RT-PCR (QRT-PCR), RNA was isolated using Trizol (Invitrogen, Carlsbad, California), treated with DNase I and purified again using RNeasy (Qiagen, Valencia, California). QRT-PCR was done essentially as previously described[22]. All samples ($n = 4$ HIF-1α, 4 ZFP, 8 contralateral controls) were amplified in duplicate and values averaged for analysis. QRT-PCR of 18S RNA was used for standardization.

Ear angiogenesis and wound healing studies. Ear angiogenesis studies were a modification of an approach described previously[12]. Adenovirus vectors encoding either a *Vegfa*-activating ZFP or murine $Vegfa_{164}$ were injected subcutaneously into the ears of CD-1 mice (10^8 PFU in 15 μl). Contralateral ears were injected with adeno-GFP. Digital photographs were obtained after 3 or 6 d. Evans blue dye (200 μl of 4% solution) was injected in the tail vein and the distribution in the ears was assessed and photographed 3 h later. Frozen sections (5 μm) were immunostained with a monoclonal anti-

body against the endothelial-cell marker PECAM and vessel counts obtained as described[22]. QRT-PCR analysis was carried out with RNA isolated from ears 3 d after transduction using splice variant–specific and splice variant–nonspecific primer–probe pairs to differentiate between the amounts of total and splice-variant Vegfa mRNA.

Cutaneous wounds were created by using a 5-mm round punch bioptome essentially as described[14]. Duplicate wounds were created on contralateral flanks of CD-1 mice and treated with adenovirus encoding either mVZ-57 or GFP (10^8 PFU in 25 μl PBS). After 5 d, wounds were excised, bisected exactly in the center and embedded in paraffin. Serial 5-μm sections were stained with H&E. Re-epithelialization was assessed by measuring the distance between the leading edges of keratinocyte ingrowth on digital images. Alternate sections were immunostained and vessel counts obtained as described[22]. Studies were carried out under approved animal protocols approved by the Yale University animal care and use committee.

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Competing financial interests

The authors declare competing financial interests: see the website (http://www.nature.com/naturemedicine) for details.

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APPENDIX C

Engineered Zinc Finger–Activating Vascular Endothelial Growth Factor Transcription Factor Plasmid DNA Induces Therapeutic Angiogenesis in Rabbits With Hindlimb Ischemia

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Background—Therapeutic angiogenesis seeks to promote blood vessel growth to improve tissue perfusion. Vascular endothelial growth factor (VEGF) exists in multiple isoforms. We investigated an engineered zinc finger–containing transcription factor plasmid designed to activate the endogenous VEGF gene (ZFP-VEGF).

Methods and Results—New Zealand White rabbits (n=56) underwent unilateral femoral artery ligation and excision. At day 10 postoperatively, the ischemic muscle received ZFP treatment (500 μg ZFP-VEGF plasmid) or no ZFP treatment (β-galactosidase, empty, or no plasmid). Group 1 (n=13) was harvested 3 days after injection to examine VEGF mRNA by real-time polymerase chain reaction and protein by ELISA. Groups 2 (n=13) and 3 (n=10) were harvested 11 days after injection. Group 2 was studied by histology and group 3, by histology and changes in blood flow. Groups 4 and 5 (n=10 each) were harvested 22 and 32 days after injection, respectively, and studied for changes in blood flow. In group 1, VEGF mRNA copy numbers were significantly higher for $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and protein in the ZFP-VEGF-treatment versus no-ZFP-treatment arms. In groups 2 and 3, capillary density and proliferating cells were significantly greater and apoptosis significantly lower in the treatment versus no-treatment arms. Changes in the blood flow ratio of the ischemic to the nonischemic limb were significantly greater in the treatment versus no-ZFP-treatment groups (6.57±1.52% versus 3.38±0.87%, $P<0.005$; 13.15±1.77% versus 6.13±1.55%, $P<0.001$; and 20.16±2.84% versus 13.88±3.14%, $P<0.01$, for groups 3, 4, and 5, respectively).

Conclusions—This engineered ZFP-VEGF–activating transcription factor may provide a novel approach to treat peripheral arterial disease. (***Circulation.*** **2004;110:2467-2475.**)

Key Words: muscle ■ angiogenesis ■ growth substances ■ apoptosis ■ endothelium-derived factors

Peripheral arterial obstructive disease (PAOD) due to atherosclerotic vascular disease is a major health problem in the United States. The 2 primary clinical manifestations of PAOD are intermittent claudication and critical limb ischemia. With intermittent claudication, there is leg pain or aching with exercise that is relieved with rest, and in critical limb ischemia, there is rest pain, ischemic ulcers, or gangrene.[1] Collectively, PAOD has an age-adjusted prevalence of 12%, and the number of patients with PAOD will increase as the population ages.[1–3] Although the primary pathophysiology of PAOD is obstructive atherosclerosis leading to impaired perfusion in the lower extremities, treatment strategies in patients with PAOD are directed toward modifying the underlying pathophysiological etiologies.[4] Surgical or percutaneous revascularization strategies may be able to improve blood flow in selected patients with PAOD, but a large number of patients with PAOD are not eligible or are poor candidates for revascularization.[5] Currently, there are no medical treatments for PAOD designed to increase blood flow to the ischemic limb.[5]

Angiogenesis is defined as the growth and development of new capillaries from preexisting vasculature, and therapeutic angiogenesis seeks to exploit this phenomenon for the treatment of disorders of inadequate tissue perfusion.[6,7] The exogenous administration of an angiogenic growth factor offers promise for patients with PAOD. Vascular endothelial growth factor (VEGF) is a prototypical angiogenic growth factor that stimulates angiogenesis in vitro and in vivo.[8]

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TABLE 1. Summary of Groups Used in Analyses (N=56 Rabbits)

Group 1 (n=13): Harvesting at Day 3 After Injection; Total Duration 13 Days	Group 2 (n=13): Harvesting at Day 11 After Injection; Total Duration 21 Days	Group 3 (n=10): Harvesting at Day 11 After Injection; Total Duration 21 days	Group 4 (n=10): Harvesting at Day 22 After Injection; Total Duration 32 Days	Group 5 (n=10): Harvesting at Day 32 After Injection; Total Duration 42 Days
VEGF expression	...	...	...	...
...	Histology	Histology	Histology	Histology
...	Blood flow measurement (only at day 21 of ischemia before euthanasia)	Blood flow measurement at days 10 and 21	Blood flow measurement at days 10 and 32	Blood flow measurement at days 10 and 42
n=6 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)	n=8 ZFP-VEGF treated (plasmid ZFP-VEGF/saline)	n=5 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)	n=3 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)	n=5 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)
n=7 Control ("empty" ZFP plasmid)	n=5 Control (plasmid pCMV-β-gal/saline)	n=5 Control (plasmid pCMV-β-gal/saline and sham)	n=7 Control (empty plasmid and poloxamer only)	n=5 Control (saline only)

All rabbits received injection materials at day 10 of ischemia (amount of injected material in a total volume of 2 mL: 500 μg for plasmids). CMV indicates cytomegalovirus; β-gal, β-galactosidase. All other abbreviations are as defined in text.

Alternative splicing of the human VEGF mRNA produces at least 4 protein isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, that vary with respect to their solubility and affinity for binding to extracellular matrix.[8,9] All of these VEGF isoforms exist in vivo, and the various isoforms appear to be differentially regulated in the setting of ischemia.[10]

The delivery of a single isoform of VEGF has been used in a number of uncontrolled human clinical trials that reported exciting clinical results, although significant edema developed in the VEGF-treated limb.[11–13] VEGF has been used in only 1 placebo-controlled clinical trial in patients with PAOD. The RAVE trial used unilateral intramuscular administration of adenovirus encoding $VEGF_{121}$, and in that study, $VEGF_{121}$ was not associated with improved exercise performance or quality of life but was associated with increased peripheral edema.[14] Taken together, human investigations that have used a single isoform of VEGF have been disappointing.

A number of genes, including the VEGF gene, contain DNA sequences that serve as sites for the binding of endogenous zinc-finger transcription factors, and some of these zinc-finger binding sites have the potential to serve as sites that regulate gene expression in vivo.[15–18] Zinc-finger protein (ZFP) transcription factors can also be engineered to bind to specific DNA sequences, and by fusing the DNA binding protein to a transcriptional activator or repressor, these engineered transcription factors can be used to increase or decrease, respectively, gene expression. Rebar et al[16] showed that an engineered ZFP linked to a transcriptional activation domain was able to increase endogenous VEGF gene expression in normal mouse skeletal muscle and promote angiogenesis in a mouse ear model in vivo. When compared with the delivery of a single VEGF isoform, the potential advantages of a gene therapy approach with a ZFP transcription factor is that activation of the endogenous VEGF gene should produce multiple VEGF splice variants. However, it was unknown whether a VEGF-activating transcription factor could induce gene expression and favorably modulate the angiogenic response in ischemic muscle. To address this question, we used a rabbit hindlimb ischemia model to test the effects of intramuscular delivery of a plasmid DNA encoding a ZFP-VEGF–activating transcription factor.

Methods

Plasmid DNA Constructs and Formulations

The VEGF-activating ZFP (mVZ+426) was provided by Sangamo BioSciences (Point Richmond, Calif) and has been previously described.[16] In brief, the genetically engineered plasmid encodes the designed 3-finger ZFP DNA-binding domain, the nuclear translocation signal from simian virus 40 large T antigen, and the transactivation domain from the p65 subunit of the human nuclear factor-κB, subcloned into pVAX1 (Invitrogen) with expression under direction of the cytomegalovirus promoter. An identical plasmid lacking the ZFP insert and a plasmid DNA encoding the β-galactosidase gene were also provided and previously described.[16] Vials containing the ZFP-VEGF plasmid, the identical plasmid without the insert, and the β-galactosidase were identical in appearance and were coded to maintain blinding until data analysis was complete.

Hindlimb Ischemia Model and Treatment Groups

In total, 56 female New Zealand White rabbits with surgically induced unilateral hindlimb vascular insufficiency were studied. By previously described methods,[16,19] rabbits (mean weight, 3.1 kg) were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). A longitudinal incision was made along the left medial thigh to allow proper isolation, ligation, and excision of the femoral artery from its origin just above the inguinal ligament to its bifurcation at the origin of the saphenous and popliteal arteries. The inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries were also isolated and ligated. The incision was closed and all animals were closely monitored during the postoperative period. Two investigators (Q.D. and J.H.) performed all of the surgical procedures.

After 10 days of ischemia, the rabbits were again anesthetized with ketamine and xylazine. For some groups (Table 1), bilateral hindlimb perfusion measures were performed, and these methods are described in a subsequent paragraph. At this time, all rabbits were divided into "ZFP-treatment" or "no-ZFP-treatment" arms. A separate 2-cm longitudinal incision was made along the ischemic limb to allow exposure of the anterior leg, tibialis anterior (TA) muscle belly. In all cases, 4 intramuscular injections were made with a 27G1/2 syringe needle evenly across the muscle. Each injection was performed smoothly for at least 15 seconds, and the needle was left in place for at least 10 seconds to prevent backflow of the injected material. The skin incision was then closed with 2-0 interrupted silk sutures (Ethicon). Group assignments were made to accomplish

different study objectives and therefore, had different lengths of time from dosing until study termination. Based on their group assignment (Table 1), the study was completed 3 days later (group 1, n=13), 11 days later (group 2, n=13, and group 3, n=10), 22 days later (group 4, n=10), or 32 days later (group 5, n=10). Within the aforementioned groups, the ZFP-VEGF–activating transcription factor was administered as 500 μg of plasmid in a total volume of 2 mL of 1% poloxamer or saline (150 mmol/L). The no-ZFP-VEGF treatment included several complementary methods: a plasmid encoding a cytomegalovirus β-galactosidase in saline (500 μg in a total volume of 2 mL), an "empty" plasmid that contained the same backbone as the ZFP-VEGF but no insert (500 μg in a total volume of 2 mL), poloxamer with no plasmid DNA (500 μg in a total volume of 2 mL), saline only (total volume 2 mL), and sham (needle-only) injections. A single investigator (Q.D.) who, with the exception of the 2 rabbits within group 3 in which no material was injected, was blinded to the "ZFP-VEGF treatment" or "no-ZFP-VEGF treatment" arm assignment, performed all of the treatments. All protocols and procedures involving animals conformed with the Guidelines for Use of Laboratory Animals published by the US Department of Health and Human Services and approved by the Duke University Animal Care and Use Committee. All animals received care in accordance with *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Tissue Procurement, Histological Section Preparation, and mRNA and Protein Extraction

After bilateral hindlimb perfusion measures were performed after light anesthesia was induced with ketamine and xylazine, additional anesthetic was given, the ischemic and contralateral nonischemic TA muscles were surgically excised from tendon to tendon, and the rabbits were euthanized after muscle extraction with a single intravenous dose of phenobarbital (150 mg/kg) and phenytoin sodium (25 mg/kg). The tissue sample was divided into several parts. The midportion of the muscle was placed in 30% sucrose–phosphate-buffered saline solution (PBS), mounted in cross section in OCT compound, and snap-frozen in LN_2. Cryostat sections (5 μm) were prepared on microscope slides (Superfrost Plus, Fisher Scientific) for histological analysis. The remainder was snap-frozen in LN_2 for RNA and protein extraction. Muscle samples were weighed and extracted with TRIzol total RNA isolation reagent (GIBCO-BRL). In brief, tissue samples were homogenized in 200 μL TRIzol reagent per 50 to 20 mg tissue and incubated for 10 minutes at room temperature; afterward, 40 μL chloroform per 200 μL of TRIzol reagent was added. After centrifugation at 13 000*g* for 20 minutes at 4°C, the RNA-containing colorless upper aqueous phase of the mixture was collected, precipitated, and washed with isopropyl alcohol and 75% ethanol. RNA concentration was determined by spectrophotometry. For protein determination, muscle samples were weighed, homogenized, and centrifuged, and the protein content of the supernatant was determined by Bradford assay by previously described methods.[10,19,20]

Measurement of VEGF mRNA and Protein in Skeletal Muscle

After extraction, the RNA was treated with DNase and purified with use of an RNeasy Mini kit (Quiagen) according to the manufacturer's instructions. One microgram of total RNA was used for first-strand cDNA synthesis by reverse transcription (RT) with Multi-Scribe reverse transcriptase and random-hexamer primers for 12 minutes at 42°C as described by the manufacturer's instructions (GeneAmp Gold RNA PCR reagent kit, AB Applied Biosystems). In total, 50 ng of cDNA products were amplified by quantitative TaqMan real-time RT–polymerase chain reaction (PCR) with a TaqMan Universal PCR Master Mix kit as described in the manufacturer's instructions (AB Applied Biosystems). A single-tube PCR was optimized for quantification of rabbit specific primers and probes for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ as shown in Table 2. A sequence detector (ABI Prism 7700, PE Applied Biosystems) was used to continuously measure the amplified product in direct proportion to the increase in fluorescence emission during the PCR amplification. All real-time RT-PCR data were captured with sequence detector 1.7 software (PE Applied Biosystems). For each sample, an amplification plot was generated. From each amplification plot, real threshold cycle (*Ct*) value was calculated, representing the PCR cycle number at which fluorescence was detectable above an arbitrary threshold. The target gene's mRNA concentrations were calculated with the *Ct* value and were normalized against 18S rRNA. Negative controls lacking template RNA were always included in each experiment. Each sample was tested in triplicate. Total VEGF protein concentrations were determined by a solid-state ELISA system with a Quantikine VEGF ELISA kit (R&D Systems) as previously described.[10]

TABLE 2. Summary of Groups Used in Analyses (N=56 Rabbits)

Primer and Probe Name (5′–3′)	Sequence	Product S
$VEGF_{121}$		
Forward primer	GAGATGAGCTTCCTACAGCA	77 bp
Reverse primer	TCGGCTTGTCACATTTTCTTG	
Probe	TGTCTTTCTTTGGTCTGCATTCACA	
$VEGF_{165}$		
Forward primer	AGAGCAAGGCAAGAAATCCC	90 bp
Reverse primer	TGCAGGAACATTTACACGTCT	
Probe	AAATGCTTTCTCCGCTCTGAGCAA	
$VEGF_{189}$		
Forward primer	AGGAAAGGGCAAGGGGCAA	116 bp
Reverse primer	TCTGCGGATCTTGTACAAACA	
Probe	CCCACAGGGAACGCTCCAGGA	
Rabbit 18S rRNA		...
Forward primer	TTCCGATAACGAACGAGACTCT	
Reverse primer	TGGCTGAACGCCACTTGTC	
Probe	TAACTAGTTACGCGACCCCCGAG	

Abbreviations are as defined in text.

Analysis of Capillary Density, Proliferation, and Apoptosis in Skeletal Muscle

Capillary density in skeletal muscle was measured by endogenous endothelial alkaline phosphatase staining on frozen sections by a previously described method.[10,19] In brief, slides were prefixed in acetone and incubated with nitroblue tetrazolium–5-bromo-4-chloro-3-indoylphosphate-*p*-toluidine salt (GIBCO-BRL) at room temperature for 1 hour. The slides were postfixed in 10% formalin and then counterstained with eosin. Capillaries appear dark blue against a red background. Capillary density expressed by the number of capillaries per square millimeter was measured by counting 6 random, high-power (magnification, ×200) fields or a minimum of 200 fibers from each (ischemic and nonischemic) limb on an inverted light microscope. Photographs were taken with an Optometrics analog camera and Adobe Premier Version 5.1, and these images were analyzed with an NIH Image analysis system. A hemocytometer was used to standardize area measurements. To ensure that the analysis of capillary density was not subjected to error from muscle atrophy or interstitial edema, vascular density was also determined by dividing the number of capillaries by the number of muscle fibers to yield the capillary–muscle fiber ratio.

Immunohistochemistry was performed with modifications of previously described methods.[19–21] Frozen sections were allowed to come to room temperature and placed in ice-cold acetone for 10

minutes and then in PBS for three 5-minute washes. Blocking solution (10% normal horse serum in PBS) was applied for 20 minutes at room temperature. To confirm the results of the alkaline phosphatase stains, an endothelial cell antibody (CD31, R&D Systems) was used at a final concentration of 1 μg/mL at 4°C overnight. Endothelial cells with adjacent pericytes or vascular smooth muscle cells were identified with an antibody against α-smooth muscle actin (HHF35, DAKO Corp) at a final concentration of 16.4 μg/mL at room temperature for 1 hour. To detect the fraction of proliferating cells, a mouse monoclonal anti-human proliferating cell nuclear antigen (PCNA) antibody (DAKO Corp) was used at a final concentration of 1 μg/mL at 4°C overnight. Incubation with the primary antibody was followed by sequential incubation with a biotinylated anti-mouse IgG and ABC reagent, according to the manufacturer's instructions (Vectastain ABC kit, Vector Laboratories). Levamisole was added to block endogenous alkaline phosphatase activity, and immune complexes were localized with the use of the chromogenic alkaline phosphatase substrate Vector Red (Vector Laboratories). All sections were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). For the PCNA study, antibody to human tonsil tissue was used as a positive control, and PBS was used in place of a primary antibody as a negative control.

An in situ nick end-labeling (NEL) apoptosis kit (ApopTag, Intergen Co) was used to label apoptotic cells in skeletal muscle of histological sections as described.[19,20] This method labeled the fragmented DNA with terminal deoxynucleotidyl transferase (TdT) with digoxigenin-conjugated peroxidase staining and a methyl green counterstain, as described in the manufacturer's instructions. Positive control slides were pretreated with DNase I (Sigma) at 100 ng/mL for 10 minutes at room temperature, followed by end-labeling. Negative controls were performed without active TdT. In selected samples, TUNEL staining was followed by immunohistochemistry to identify the apoptotic cell type.

For the PCNA staining and apoptosis studies, the proliferation index and apoptotic index were expressed as a percentage of the number of positive nuclei divided by the total number of nuclei. The count was performed on 3 randomly selected fields (magnification ×200) and at least 200 nuclei per sample. A single reader blinded to sample type performed all of the analyses. The variability on repeated measures was <10%.

Hemodynamic Assessment

Bilateral hindlimb perfusion was measured with VASAMEDICS LASERFLO blood perfusion monitor laser Doppler equipment (VASAMEDICS, Inc) after induction of anesthesia with ketamine and xylazine. Based on the group assignments shown in Table 1, blood flow was measured at the day of study termination or at both preinjection (10 days after ligation) and at 11, 21, or 31 days after injection, which correspond to a total of 21, 32, and 42 days of ischemia. In total, 6 sites from the upper, middle, and lower part of each hindlimb were selected for measurement for each limb. For all analyses, a mean value was calculated from all 6 measurements per limb.

Statistical Analysis

Unless otherwise stated, results are expressed as mean±SD, the only exception being for VEGF expression data, for which data are expressed as mean±SE. Statistical significance was evaluated by Student's *t* test for paired or unpaired variables. $P<0.05$ or less was considered statistically significant.

Results

Treatment With the ZFP-VEGF Plasmid Increased VEGF mRNA Expression and Protein Production in Ischemic Skeletal Muscle

First, we sought to determine whether the ZFP-VEGF plasmid was able to increase the expression of 3 different VEGF splice variants and VEGF protein. In total, 13 rabbits were harvested on day 3 after injection (total of 13 days of ischemia) from group 1 (Table 1). This ZFP plasmid dose was selected from studies performed in nonischemic rabbit muscle (J.R., unpublished data), and the 3-day postinjection time was selected because that was when the plasmid was expected to be expressed but before changes in muscle blood flow would occur, as the latter could influence VEGF expression. The no-ZFP-treatment arm used the plasmid without the ZFP-VEGF insert.[16] Expression of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNAs was measured by quantitative real-time RT-PCR and expressed as mRNA concentration in the ischemic limb minus the value in the nonischemic limb for each animal. As shown in Figure 1A (top), the differences were 5.65±2.79, 3.99±2.07, and 5.67±2.81 in the ZFP-VEGF–treated arm compared with 0.12±0.24, −0.20±0.19, and −0.26±0.19 in the no-ZFP-treatment arm for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNAs ($P=0.05$, $P=0.05$, and $P=0.04$), respectively. VEGF protein was measured by ELISA and was expressed as concentration in the ischemic limb minus the nonischemic limb for each animal. As shown in Figure 1B (bottom), the difference in VEGF protein was 40.65±8.83 versus 9.06±8.15 pg/mg soluble protein ($P=0.02$) for the ZFP-treatment versus no-ZFP-treatment arms. The mRNA data were also analyzed as the difference in VEGF mRNA in the ischemic limb of the ZFP-treatment arm compared with the no-ZFP-treatment arm, and the values were 15.05±8.58 versus 1.80±0.27, 12.57±7.04 versus 1.20±0.27, and 10.19±5.0 versus 1.08±0.26 for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNA, respectively. These differences were not statistically different; however, levels of VEGF protein were significantly greater in ischemic muscle in the ZFP-treatment arm versus the no-ZFP-treatment arm (88.9±10.75 versus 52.31±7.07 pg/mg soluble protein, $P=0.01$). Taken together, these data suggest that the ZFP-VEGF transcription factor was able to increase VEGF mRNA and protein expression in ischemic skeletal muscle.

ZFP-VEGF Plasmid Favorably Modulates Capillary Density, Cell Proliferation, and Apoptosis in Ischemic Skeletal Muscle

Next, we sought to determine whether the ZFP-VEGF plasmid was able to increase vascular density in ischemic skeletal muscle compared with no ZFP treatment 11 days after injection. Alkaline phosphatase staining was used to measure capillary density, and representative examples are shown in Figure 2A and 2B. As shown in Table 1, a total of 23 rabbits from groups 2 and 3 were available for quantitative analysis (n=13 in the ZFP-VEGF treatment arm and n=10 in the no-ZFP-treatment arm). As shown in Figure 2C, capillary density in the ischemic TA muscle was significantly higher in the ZFP-VEGF-treatment arms compared with the no-ZFP-treatment arm (310±71 versus 198±25 capillaries/mm^2, $P<0.001$). To exclude any potential differences induced by changes in muscle fiber diameter, the capillary–muscle fiber ratio in the ischemic TA muscle was measured. The capillary–muscle fiber ratio was also significantly higher in the ZFP-VEGF-treatment arm compared with the no-ZFP-treatment arm (1.08±0.15 versus 0.85±0.11, $P<0.05$). By CD31 immunostaining, capillary density was 1.9-fold higher

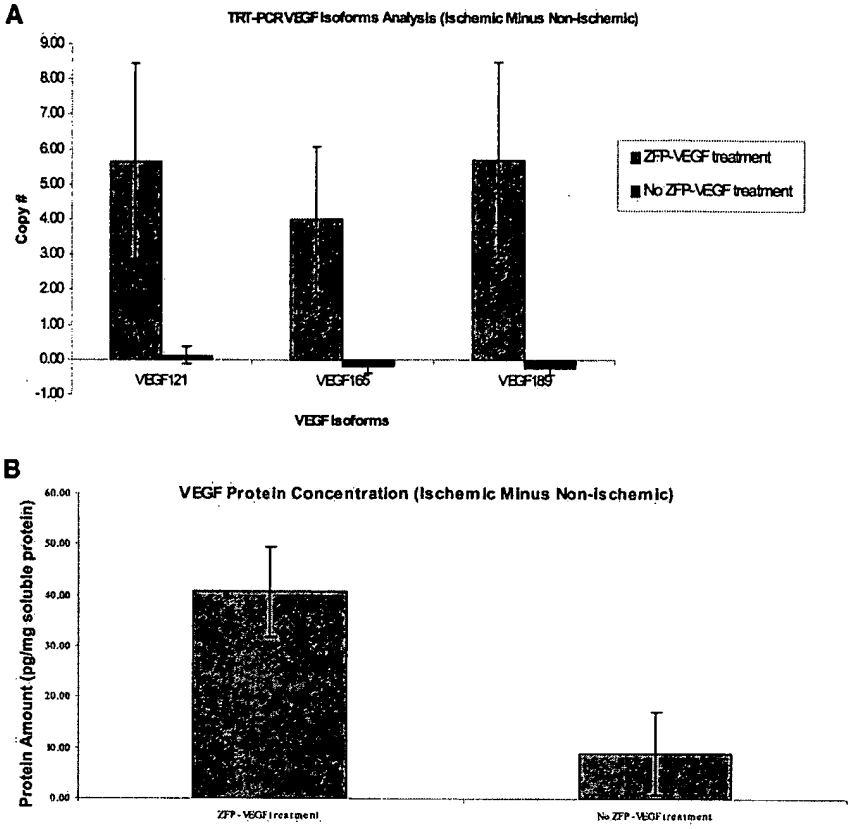

Figure 1. A, VEGF mRNA expression for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ by quantitative real-time RT-PCR in group 1 (day 3 after injection; n=6 in ZFP-VEGF–treated arm and n=7 in no-ZFP-VEGF-treatment arm). VEGF expression was measured as copy number of each isoform in ischemic leg minus that in nonischemic limb for each animal. Expression for all 3 mRNA isoforms was significantly higher (P=0.05, P=0.05, and P=0.04 for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, respectively) with ZFP-VEGF treatment compared with no ZFP-VEGF treatment. B, VEGF protein expression was measured by ELISA and was found to be significantly greater with ZFP-VEGF treatment compared with no ZFP-VEGF treatment (P=0.02). Abbreviations are as defined in text.

in the ZFP-VEGF-treatment arm (1.12±0.42) compared with the no-ZFP-treatment arm (0.59±0.39, P<0.03).

We then sought to determine whether changes in proliferation, cell death, or both contributed to the changes in vascular density in the ischemic muscle treated with the ZFP-VEGF plasmid. Representative samples of PCNA and TUNEL staining are shown in Figure 3. For quantitative assessment, the same 23 samples used in the vascular density studies were available. As shown in Figure 4A, the number of PCNA-positive cells in the ischemic TA muscle was significantly greater in the ZFP-VEGF-treatment arm (3.11±1.10%) compared with the no-ZFP-treatment arm (0.49±0.62%, P<0.01). Within the ZFP-VEGF treatment arm, the number of PCNA-positive cells in the ischemic limb was significantly greater than in the contralateral, nonischemic, noninjected TA muscle (3.11±1.10% versus 1.72±1.01%, P<0.01). As shown in Figure 4B, the number of TUNEL-positive nuclei in ischemic TA muscle was significantly lower in the ZFP-VEGF-treatment arm (1.02±0.54%) compared with the no-ZFP-treatment arm (1.90±0.68%, P<0.01). Within the ZFP-VEGF-treatment arm, the number of TUNEL-positive nuclei in the ischemic limb was similar to that in the contralateral, nonischemic, noninjected muscle.

Effects of ZFP-VEGF Plasmid at Later Times

Rabbits from group 3 (11 days after injection, 21 days of total ischemia), group 4 (21 days after injection, 32 days of total ischemia), and group 5 (21 days after injection, 32 days of total ischemia) underwent blood flow measurements before injection and before sacrifice (Table 1). Perfusion in the ischemic limb was measured as a percentage of that in the nonischemic limb at both times. Immediately before injection, the mean perfusion ratio was 61.7±2.8% for all rabbits, and there was no difference in these baseline values in either arm (62.2±2.9% for ZFP treatment versus 61.3±2.9% for no ZFP treatment, P=NS). As shown in Figure 5A, the change in perfusion from 10 to 21 days was 6.57±1.52% in the ZFP-treatment arm versus 3.38±0.87% in the no-ZFP-treatment arm (P<0.01). As shown in Figure 5B, the change from 10 to 32 days was 13.15±1.77% in the ZFP-treatment arm versus 6.13±1.55% in the no-ZFP-treatment arm (P<0.001). As shown in Figure 5C, the change from 10 to 42 days was 20.16±2.84% in the ZFP-VEGF-treatment arm versus 13.88±3.14% in the no-ZFP-treatment arm (P<0.01). Qualitatively, similar results were obtained when changes in absolute perfusion values to the ischemic limb were used in place of the ratio. In groups 4 and 5, the number of α-smooth muscle actin–positive blood vessels per square millimeter was greater in the ZFP versus no-ZFP treatment arms (68.3±37.0 versus 43.2±21.1, P=0.05).

Discussion

Angiogenesis is a complex process whereby new blood vessels are formed from preexisting vascular structures.[6] Therapeutic angiogenesis seeks to exploit the phenomenon of angiogenesis to treat disorders of inadequate tissue perfusion, such as in patients with ischemic heart or PAOD.[7] VEGF has been and continues to be a leading candidate molecule for therapeutic angiogenesis. Although VEGF exists in multiple

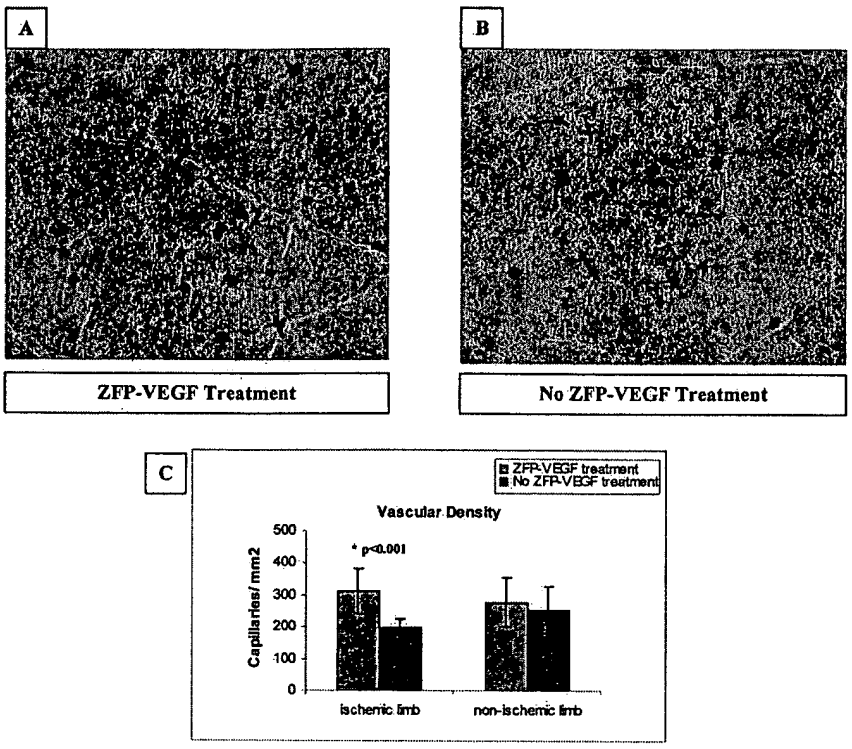

Figure 2. Representative alkaline phosphatase stains show that capillary density in ischemic TA muscle with ZFP-VEGF treatment (A) was higher than with no ZFP-VEGF treatment (B) at 11 days after injection. Dark blue dots indicate capillaries (magnification ×200). C, Quantitative assessment of capillary density (capillaries/mm^2) in ischemic TA muscle (310±71/mm^2) with ZFP-VEGF treatment was significantly higher than that of no ZFP-VEGF treatment (198±25/mm^2). *P<0.001 vs ZFP-VEGF treatment. Abbreviations are as defined in text.

isoforms, approaches to achieve therapeutic angiogenesis in humans have exclusively used a single isoform of VEGF delivered as a protein, a plasmid-encoding cDNA, or an adenovirus.[12,14,22–24] The results from placebo-controlled trails with VEGF have been disappointing.[12,22] A method that would enhance the production of multiple VEGF isoforms has theoretical advantages over single-isoform therapy and thereby could overcome the limitations found in early human studies.

In this report, we sought to evaluate the effects of intramuscular injection of a plasmid DNA encoding a ZFP-VEGF–activating transcription factor in a preclinical model of PAOD. There were several notable findings. First, the ZFP-VEGF transcription factor led to upregulation of VEGF protein and 3 different VEGF isoforms in ischemic skeletal muscle. Second, treatment with the ZFP-VEGF plasmid led to an angiogenic response with an increase in capillary density, an increase in cellular proliferation, and a reduction in apoptosis in the ischemic TA muscle. Finally, there was evidence of a therapeutic angiogenic effect with an increase in perfusion to the ischemic limb in the ZFP-VEGF compared with the no-ZFP-VEGF treatment arms. These data demonstrate for the first time that modulation of endogenous VEGF gene expression and thereby, multiple VEGF isoforms can lead to therapeutic angiogenesis in a preclinical model of PAOD.

Zinc-finger transcription factors function by binding to specific DNA sequences in the regulatory region of a target gene. Rebar et al[16] found that the same ZFP-VEGF plasmid used in our current study was able to promote angiogenesis in a mouse ear. In ischemic muscle, endogenous transcription factors could have been present in the regulatory portion of the VEGF gene that contained the DNA sequences that served as the binding site for the engineered transcription factor, and this would have resulted in the ZFP-VEGF not being able to increase VEGF mRNA or protein. This was not the case, and the ZFP-VEGF–activating transcription factor was able to increase VEGF gene expression in ischemic peripheral skeletal muscle.

Although the 3 VEGF isoforms examined in our report were upregulated at the mRNA level in ischemic muscle after ZFP-VEGF treatment, the increase in the $VEGF_{189}$ is particularly noteworthy. Differential splicing of the VEGF mRNA transcript results in isoforms that range in length from 121 to 206 amino acid residues.[8,9] The $VEGF_{121}$ isoform does not bind to the extracellular matrix and therefore, is freely soluble. $VEGF_{165}$ displays some heparin-binding properties and can also be detected in the circulation. $VEGF_{189}$ and $VEGF_{206}$ bind with high affinity to heparin sulfate proteoglycans in the extracellular matrix, which likely results in greater tissue retention of VEGF protein. It is interesting to speculate that an increase in expression of the higher-molecular-weight VEGF isoforms might yield better effects than either a single isoform or the lower-molecular-weight isoforms. This possibility is supported by recent work of Whitlock et al,[25] in which adenovirally mediated coexpression of 3 different VEGF splice variants ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$) in 1 virus with a single insert encoding multiple isoforms or a mixture of 3 different viruses in a mouse model of hindlimb ischemia was superior in restoring blood than any one isoform alone. As previously noted, in a mouse ear model, Rebar et al[16] demonstrated that the same ZFP-VEGF–activating transcription factor used in our current report led to marked angiogenesis; however, unlike treatment with $VEGF_{164}$ (the murine variant) alone, the ZFP-VEGF plasmid–enhanced angiogenesis occurred without increasing vascular

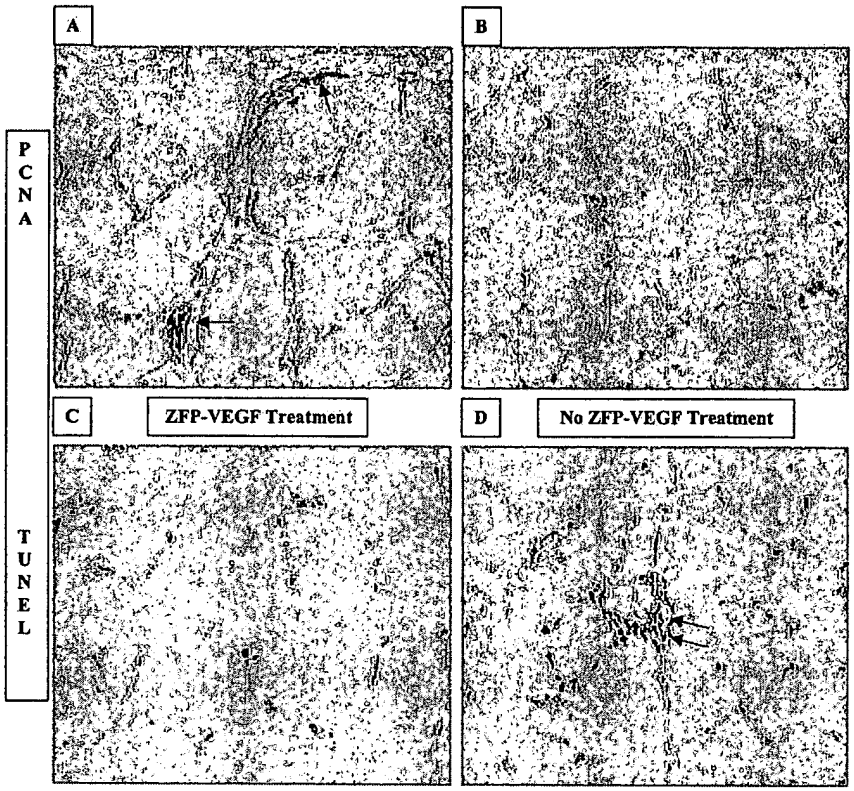

Figure 3. Representative examples of PCNA immunohistochemistry (A and B) and TUNEL staining (C and D) for labeling of proliferating and apoptotic nuclei from ischemic muscle from ZFP-VEGF-treatment (A and C) and no-ZFP-VEGF-treatment (B and D) arms at 11 days after injection. PCNA-positive cells (red, arrow) are visible in ischemic muscle with ZFP-VEGF treatment (A) but are rarely seen in ischemic muscle with no ZFP-VEGF treatment (B). TUNEL-positive nuclei (brown, arrow) are visible in ischemic muscle from both groups, but number of apoptotic cells in ZFP-VEGF–treated sample (C) was lower than that with no ZFP-VEGF treatment (D). Magnification, ×200 for all panels. Abbreviations are as defined in text.

leakiness. Vascular maturation is a complex process that involves the recruitment of nonendothelial support cells such as pericytes and vascular smooth muscle cells, both of which express smooth muscle actin.[26] In our report, treatment with the ZFP-VEGF–activating plasmid did result in an increase in the number of smooth muscle actin cells at 22 and 32 days after injection, although effects on vascular permeability after treatment of ischemic muscle were not examined.

We previously demonstrated that hindlimb ischemia results in a decrease in proliferation and an increase in apoptosis in ischemic muscle at 21 days after ligation, and the majority of apoptotic cells were endothelial.[10,19] In group 2 in our current study, we found that treatment of ischemic muscle with the ZFP-VEGF–activating transcription factor resulted in an increase in the fraction of PCNA-positive cells along with a decrease in the fraction of apoptotic cells. In the no-ZFP-VEGF-treatment arm in group 2, the majority of TUNEL-positive cells colocalized with endothelial cells (data not shown), and the fraction of apoptotic cells in the ZFP-VEGF-treatment arm was not different from that found in the nonischemic limb. This suggests an effect of VEGF on endothelial cells in ischemic skeletal muscle and confirms other reports wherein VEGF has been shown to act as a survival factor to protect endothelial cells from death.[27] Germani et al[28] showed that ex vivo treatment of myoblasts with a $VEGF_{165}$ adenovirus reduced apoptosis in vitro, and in a mouse hindlimb model, pretreatment with intramuscular adeno-$VEGF_{165}$ injection reduced apoptosis in muscle after ligation. The potential sequelae that could result from favorably modulating apoptosis in endothelial and/or other cells in ischemic peripheral skeletal muscle remain speculative.

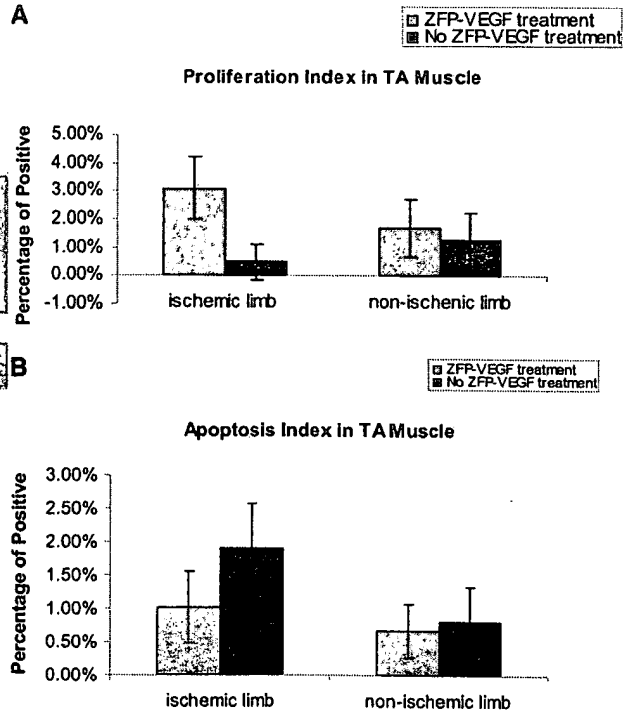

Figure 4. A and B, Results of quantitative assessments of PCNA and TUNEL staining, respectively. As shown in A, number of PCNA-positive cells in ischemic TA muscle was significantly greater in ZFP-VEGF-treatment arm (3.11±1.10%) than in no-ZFP-VEGF-treatment arm (0.49±0.62%, $P<0.01$ vs ZFP-VEGF treatment). As shown in B, number of TUNEL-positive nuclei in ischemic TA muscle with ZFP-VEGF treatment was significantly lower compared with that after no ZFP-VEGF treatment (1.02±0.54% vs 1.90±0.68%, $P<0.01$ vs ZFP-VEGF treatment arm). Abbreviations are as defined in text.

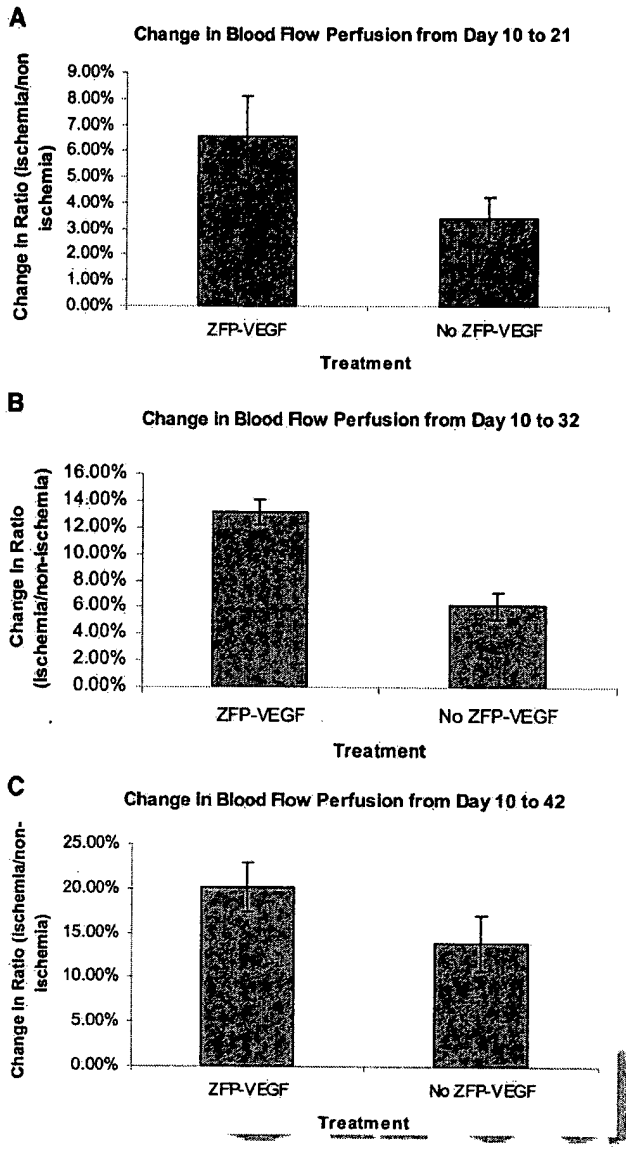

Figure 5. Treatment of ischemic hindlimb muscle with ZFP-VEGF–activating transcription factor increased perfusion at multiple times. Animals underwent laser Doppler readings on day 10 (day of ZFP treatment or no ZFP treatment) and again at study termination after 21 (A), 32 (B), and 42 (C) days of ischemia. Change in blood flow to ischemic limb in ZFP-VEGF–treated arm was significantly greater than that in no-ZFP-VEGF treated arm at all times. Abbreviations are as defined in text.

Finally, we sought to determine whether ZFP-VEGF treatment would lead to therapeutic angiogenesis as measured by improvements in blood flow in the ischemic limb. By laser Doppler measurement, we found that ZFP-VEGF treatment resulted in a significantly greater increase in perfusion at all times measured (11, 22, and 32 days after injection) when compared with no ZFP-VEGF treatment. Although it is very difficult to compare studies in the same animal model from laboratory to laboratory, the results of our study certainly support the potential efficacy of the ZFP-VEGF–activating transcription factor. When given only 2 days after surgical ligation, Gowdak et al[29] showed that treatment with 1 mg $VEGF_{165}$ plasmid DNA formulated in liposomes led to significant increases in blood flow in a rabbit hindlimb model by 11 days after treatment. When $VEGF_{165}$ was administered as an adenovirus 10 days after surgical ligation (the same time used in our report) and was compared with β-galactosidase, Vajanto et al[30] showed no significant increase in blood flow until the 30-day postinjection time in rabbits.

Our study suggests the potential of using ZFP-VEGF gene transfer to treat PAOD. Therapeutic modalities in humans must balance risks and benefits. ZFP transcription factors can be designed to target unique sites in the genome, and when studied in cell culture, they may regulate the intended target gene and no other.[31] The ZFP-VEGF used in our study targets a 9-bp sequence and is therefore predicted to have multiple potential binding sites. In cell culture, in addition to upregulation of the VEGF splice variants, other genes are upregulated at the mRNA level by microarray analysis, but VEGF is activated to the highest level.[16] The vector encoding the ZFP-VEGF–activating transcription factor could be modified with additional regulatory elements to permit changes in expression in response to intermittent stimuli (ie, hypoxia). The potential deleterious effects of prolonged VEGF expression in muscle must also be considered; however, the changes in VEGF expression from plasmid-based gene transfer are likely to be limited to period of only a few weeks.[32] likely to be limited to period of only a few weeks.[32]

Acknowledgments

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Disclosure

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APPENDIX D

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Elevation of seed α-tocopherol levels using plant-based transcription factors targeted to an endogenous locus

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Abstract

Synthetic zinc finger transcription factors (ZFP-TFs) were designed to upregulate the expression of the endogenous *Arabidopsis* γ-tocopherol methyltransferase (GMT) gene. This gene encodes the enzyme responsible for the conversion of γ-tocopherol to α-tocopherol, the tocopherol species with the highest vitamin E activity. Five three-finger zinc finger protein (ZFP) DNA binding domains were constructed and proven to bind tightly to 9 bp DNA sequences located in either the promoter or coding region of the GMT gene. When these ZFPs were fused to a nuclear localization signal and the maize C1 activation domain, four of the five resulting ZFP-TFs were able to upregulate the expression of the GMT gene in leaf protoplast transient assays. Seed-specific expression of these ZFP-TFs in transgenic *Arabidopsis* produced several lines with a heritable elevation in seed α-tocopherol. These results demonstrate that engineered ZFP-TFs comprised of plant-derived elements are capable of modulating the expression of endogenous genes in plants.

1. Introduction

The tools of plant biotechnology allow the engineering of novel traits via introduction of foreign genes into plants and expression of these genes in a developmental or tissue specific manner using selected promoters. Desired traits can alternatively be obtained by regulating the expression of endogenous genes. One method to achieve this type of targeted gene regulation is through the use of engineered transcription factors. Transcription factors are trans-acting proteins that bind to specific cis-elements and regulate gene expression. Transcription factors are typically modular, consisting of a DNA-binding domain (DBD) and an effector domain (ED) that interacts with other regulatory proteins to either activate or repress transcription. The Cys_2–His_2 zinc finger proteins (ZFPs) are the most common DBDs in eukaryotes and over the past decade this motif has emerged as amenable to manipulations designed to achieve the specific recognition of a predetermined DNA sequence (Pabo et al., 2001, Segal and Barbas, 2001, Beerli and Barbas, 2002). These designer ZFPs have been fused to different EDs to create hybrid zinc finger protein transcription factors (ZFP-TFs) that have been used successfully in the regulation of endogenous chromosomal genes in both animal cell lines (Beerli et al., 2000; Zhang et al., 2000; Bartsevich and Juliano, 2000; Dreier et al., 2001 ; Liu et al., 2001, Ren et al., 2002) and more recently transgenic plants (Guan et al., 2002).

This study reports the production of ZFP-TFs comprised of plant-derived DNA sequences and engineered to activate the endogenous *Arabidopsis*

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γ-tocopherol methyltransferase (GMT) gene (GenBank Accession AF104220). GMT encodes the enzyme responsible for addition of a methyl group to ring carbon 5 of γ-tocopherol to form α-tocopherol (Fig. 1), the tocopherol isoform with the highest vitamin E activity (Bramley et al., 2000). GMT is frequently limiting in seed tissue and as a result the tocopherol composition of seed from many plant species is made up predominantly of γ-tocopherol (Sheppard et al., 1993). Transgenic overexpression of an *Arabidopsis* GMT cDNA was previously found to result in a large increase in α-tocopherol relative to control (Shintani and Della-Penna, 1998).

Five three-finger ZFPs were designed to bind to target 9 bp sequences found in the promoter or coding region of the endogenous *Arabidopsis* GMT gene. These ZFPs were each fused to the maize opaque-2 nuclear localization signal (GenBank Accession M29411) and the maize C1 (GenBank Accession TVZMMB) activation ED (Guyer et al., 1998, Goff et al., 1991) to make ZFP-TFs. Expression of these ZFP-TFs in transgenic *Arabidopsis* under the control of an embryo specific promoter (Kridl et al., 1991) resulted in several lines that had an elevated seed α-tocopherol percentage, with the best line demonstrating a heritable 20 fold increase in percent α-tocopherol relative to control seed. Transgenically modulating endogenous gene expression to achieve a desired phenotype using ZFP-TFs offers a powerful tool to plant biotechnologists (Guan et al., 2002, Ordiz et al., 2002). This is first time that ZFP-TFs derived wholly from plant sequences have been described and found to modulate expression of an endogenous target gene in a whole plant.

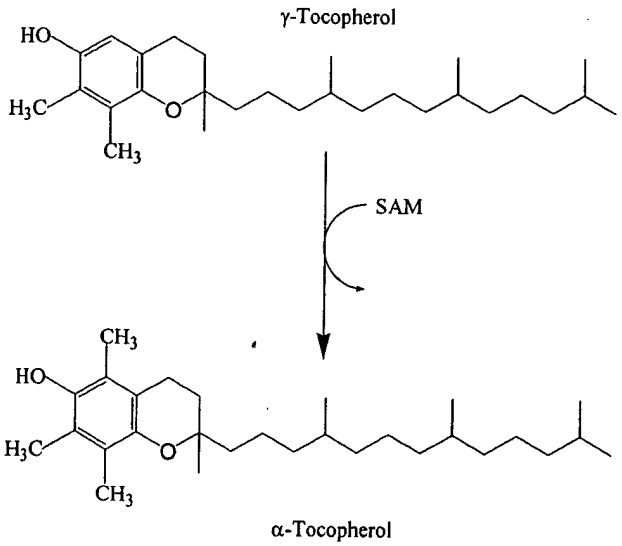


Fig. 1. Biosynthetic reaction catalyzed by GMT. GMT adds a methyl group to ring carbon 5 of γ-tocopherol.

2. Materials and methods

2.1. Nuclei isolation and DNase I hypersensitive mapping

Leaves from 3–4 week *Arabidopsis thaliana* (ecotype Columbia) grown on soil under a 16-h-light/8-h-dark cycle and 25°C constant temperature were harvested and intact nuclei were digested with increasing concentrations of DNase I (Li et al., 1998). Hypersensitivity mapping was performed essentially as described (Zhang et al., 2000). Briefly, DNA from DNase I-treated nuclei was isolated and digested to completion with Dra II, resolved on agarose gels and transferred to nylon membranes. These membranes were hybridized to an indirect end-labeled 500 bp probe derived from the genomic sequence located 1.5–2 kb upstream of the GMT transcription start site.

2.2. Synthesis, purification, and gel shift analysis of plant-derived ZFPs

The strategy used to design, synthesize and purify the ZFP DNA binding domains was as described in Jamieson and Li (2002) and Rebar and Jamieson (2002). The quantitative electrophoretic gel mobility shift assay was performed as described previously (Liu et al., 2001) except that an additional 150 mM NaCl was added to the binding buffer.

2.3. Construction of ZFP-TFs for protoplast based transient assay

ZFP binding domains were subcloned into a plant ZFP expression vector, YCF4, generated from pcDNA3.1 (Invitrogen, Carlsbad, CA). YCF4 contains a CaMV35S promoter driving expression of the coding sequences from the maize opaque-2 nuclear localization signal (RKRKESNRESARRSRRSRYRKKV) and 60 amino acids from the maize C1 activation domain (AGSSDDCSSAASVSLRVGSHDEPCFSGDGDGD WMDDVRALASFLESDEDWLRCQTAGQLA). All of the ZFP-TF expression vectors were constructed by subcloning the ZFP fragments into the KpnI and BamHI sites in YCF4 between the NLS and the C1-ED (Fig. 4A).

2.4. Isolation and transformation of Arabidopsis leaf protoplasts

Isolation and transformation of *Arabidopsis* leaf protoplasts were carried out as described (Abel and Theologis, 1994) with the following modifications. Purified protoplasts were resuspended to a density of 5×10^6 protoplast/ml in a solution containing 400 mM mannitol, 15 mM $MgCl_2$ and 5 mM Mes-KOH at pH 5.6. Approximately 1.6×10^6 protoplasts (300 μl

suspension) were added to a mixture of 50 μg ZFP-TFs plasmid DNA in 15 ml disposable conical tube and mixed well. PEG-CMS solution (400 mM mannitol, 100 mM $Ca(NO_3)_2$, 40% PEG3350) was immediately added to a final PEG concentration of 20% and the suspension was carefully mixed to a homogeneous phase. Following incubation at room temperature for 30 min, the transfected mixture was diluted by 10 ml of protoplast growth medium (400 mM sucrose, 4.4 g/L Murashige and Skoog salt and vitamin mixture (Gibco, Rockville, MD), and 250 mg/L xylose) and transferred to a 10 cm petri dish and incubated in the dark at 25°C for 18–24 h.

2.5. Analysis of endogenous GMT activation: TaqMan analysis

For TaqMan quantitative RT-PCR analysis of mRNA abundance in protoplasts, total RNA from transfected *Arabidopsis* leaf protoplast was isolated using the plant RNeasy kit (Qiagen, Valencia, CA). Real-time PCR analysis was performed in a 96-well format on an ABI 7700 SDS machine (Perkin–Elmer, Chicago, IL) and analyzed with SDS version 1.6.3 software. RNA samples (5 ng) were mixed with 0.3 μM each primer, 0.1 μM probe, 5.5 mM $MgCl_2$ and 0.3 mM each dNTP, 0.625 unit of AmpliTaq Gold RNA polymerase (Hoffman La-Roche, Inc.), 6.25 units of Multiscribe Reverse Transcriptase, and 5 units of RNase Inhibitor in TaqMan buffer A (Perkin–Elmer). The reverse transcription was performed at 48°C for 30 min. After denaturing at 95°C for 10 min, PCR amplification reactions were conducted for 40 cycles at 95°C for 15 s and at 60°C for 1 min. The *Arabidopsis* GMT primer and probe set (AATGATCTCGCGGCTGCT, GAA TGGCTGATTCCAACGCAT, FAM-TCACTCGCT CATAAGGCTTCCTTCCAAGT-TAMRA) were used to measure the *Arabidopsis* GMT expression levels. The GAPDH primer and probe set (GATCATCAAG ATTGTATCTGATC, CGGTTCCTTCGATAACTA AGT, FAM- CAATGCCTAGTTCCCCCCAGGGGAG-TAMRA) were used to monitor the internal control GAPDH mRNA.

For TaqMan analysis of mRNA levels in developing *Arabidopsis* seed, total RNA was isolated from four developing siliques per plant. Total RNA was prepared using the SV Total RNA Isolation Kit (Promega, Madison, WI). RT-PCR was performed as described above, except that the assay consisted of 30 ng total RNA, 0.8 μM each primer, 0.15 μM probe, 10 units of Multiscribe Reverse Transcriptase, and 5 units of RNAse Inhibitor in 1X TaqMan Universal PCR Master Mix w/o AmpErase® (Perkin–Elmer). The primer and probe sets (AATGATCTCGCGGCTGCT, GAATGG CTGATCCAACGCAT, FAM-TCACTCGCTCATAA GGCTTCCTTCCAAGT-TAMRA); (TGCCAGAAC AAGAAGGGTGG, ATACCGACGCCGCCG, FAM-TCGTCCGACGACCCTGCGG-TAMRA); (CGTCC CTGCCCTTTGTACAC, CGAACACTTCACCGGA TCATT, VIC-CCGCCCGTCGCTCCTACCGAT-TA MRA); and (TGCCCCACCTTGAGACAAG, CTTG CTCTGGTTGGTGTTGCT, VIC-CCCTGGAATCT AACGGCCTTGGCA-TAMRA) were used to assay GMT, C1-ED, 18S RNA, and endogenous napin RNA, respectively. For each RNA sample, two replicates were amplified in a one-step reaction and cycle threshold values were obtained. Relative values were calculated using the comparative Ct method described in the manufacturer's User Bulletin (The Perkin–Elmer Corporation, 1997).

2.6. Plant vector construction, Arabidopsis transformation

ZFP-TFs (A-E) were subcloned downstream of the napin embryo-specific promoter (Kridl, et al., 1991) using the *Not*I and *Hin*dIII sites (Fig. 4A) in a plant transformation binary vector. These vectors and their parent (lacking a ZFP-TFs insert as a control) were electroporated into *Agrobacterium tumefaciens* strain ABI and grown under standard conditions (McBride et al., 1994), reconfirmed by restriction analysis, and transformed into *Arabidopsis* using the dipping method (Clough and Bent, 1998). Transgenic T1 plants (12–22 independent insertion events for each ZFP-TF) were grown to maturity and T_2 seed was analyzed for tocopherol content and composition using normal phase HPLC methods as described by Savidge et al. (2002). The four events with the highest seed α-tocopherol percentage from each of two ZFP-TFs (A and B) were advanced to the next generation and T3 seed from 10 individual T2 plants per event was analyzed for tocopherol content and composition.

3. Results and discussion

3.1. DNase I hypersensitive mapping of the Arabidopsis GMT gene

Previous studies indicated that chromatin organization is a determinant of ZFP-TF function within endogenous chromosome loci. The positioning of nucleosomes at endogenous loci can prevent the access of DBDs to the DNA binding site (Li et al., 2001). Targeting ZFP-TFs to an open and accessible region can greatly increase their chances of successfully regulating the target gene (Zhang et al., 2000, Liu et al., 2001). DNase I hypersensitive mapping was performed to locate the regions of the GMT gene accessible to ZFPs. Given the impracticality of obtaining large quantities of *Arabidopsis* embryo tissue, leaf tissue was used for the

DNase I hypersensitive mapping of the GMT gene. These data were then utilized to infer potential accessible sites in the embryo. A previous study demonstrated that some DNase I hypersensitive sites, especially those near the transcription start site, are conserved between different tissues and cell types (Liu et al., 2001).

The DNase I hypersensitive mapping revealed four hypersensitive sites (Fig. 2). The most accessible and therefore obvious hypersensitive site was a doublet centered at −140 bp upstream of the transcription start site. It covered the transcription start site and the entire 5′ UTR. The second hypersensitive site was centered around −600 bp. This hypersensitive site was fairly narrow but had a high intensity. The two other hypersensitive sites (−1000 bp, +500 bp) did not appear to be as accessible to the DNAase I and hence were fainter and more difficult to discern.

3.2. *Biochemical characterization of ZFPs and design of ZFP-TFs*

Five 9 bp potential binding sites were selected based on their positions relative to the hypersensitive sites and their suitability as ZFP target sequences (Fig. 3A). Two of the binding sites were located in the most accessible

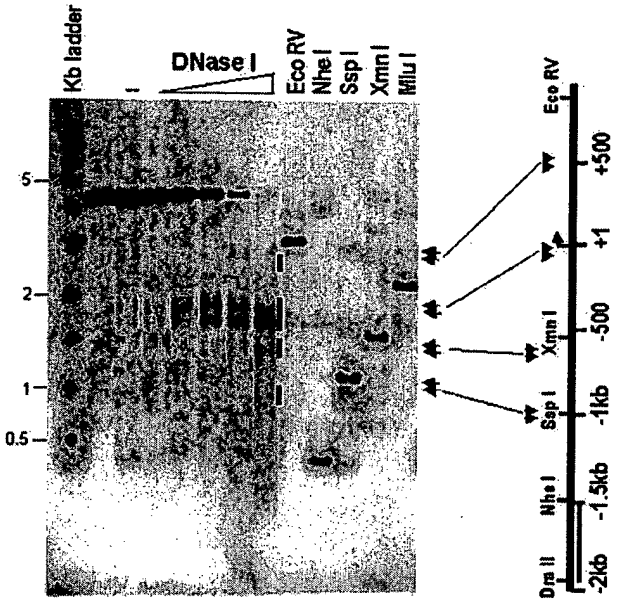
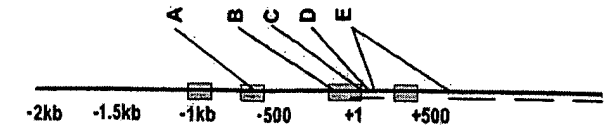


Fig. 2. DNase I digestion profile of the *Arabidopsis* GMT gene. Nuclei from *Arabidopsis* leaves were digested with increasing concentrations of DNaseI or the indicated restriction enzymes. A 500 bp sequence of the genomic sequence located 1.5–2 kb upstream of the GMT transcription start site was used as a probe on a Southern blot of extracted nuclei DNA. Transcription start site (+1) of the GMT gene (vertical graphic) and the location of the probe (black line) are shown. Double arrows indicate the relationship of the observed hypersites to the location of the region on the GMT gene.



(A)

ZFP	Target (5′ to 3′) Sequence	Subsites	Finger designs (-1 to +6)	Gel Shift	Kd (nM)
A	GAGGAAGGGg	GGGg GAAg GAGg	RSDHLAR QSGNLAR RSDNLTR	Bound Free	0.001
B	GGGGAAAGGg	AGGg GAAa GGGg	RSDHLTQ QSGNLAR RSDHLSR	Bound Free	0.0036
C	GAAGAGGGTg	GGTg GAGg GAAg	QSSHLAR RSDNLAR QSGNLAR	Bound Free	0.0036
D	GAGGAGGATg	GATg GAGg GAGg	QSSNLQR RSDNLAR RSDNLQR	Bound Free	0.0003
E	GAGGAGGAGg	GAGg GAGg GAGg	RSDNLAR RSDNLAR RSDNLTR	Bound Free	0.0008
SP1	GGGGCGGGGg	GGGg GCGg GGGg	KTSHLRA RSDELQR RSDHLSK	Bound Free	0.055

(B)

Fig. 3. The location and DNA binding properties of ZFPs targeted to the *Arabidopsis* GMT. (A) The location of the target sites of five ZFP DNA binding domains (A–E) designed to bind to 9 bp sequences in the *Arabidopsis* GMT gene. Numbering is relative to the start site of transcription (+1). Hypersensitive sites inferred from Fig. 2 (gray rectangles) and the first two exons of the GMT gene (black horizontal lines) are indicated. (B) The 9 bp target sequence, amino acid sequence of positions "−1" through "+6" of the α-helix of each zinc finger, and gel shift assays showing the apparent K_d of plant-derived zinc finger protein DNA binding domains (A–E) and SP1 (positive control) for their target sequence.

DNase I hypersensitive sites (A, B), one (C) was at the margin of a hypersensitive site, and the remainder (D and E) were not located in a hypersensitive site. We intentionally evaluated ZFPs designed to bind to sequences that were both within and external to the mapped hypersensitive sites to determine the importance of chromatin infrastructure on in planta activity of the transcription factors.

Although these five ZFPs were assembled using previous described methods (Zhang et al., 2000, Liu et al., 2001) they were distinct from any previously reported synthetic ZFPs in two ways. Firstly, the backbone of these ZFPs was of plant origin having been assembled of DNA fragments derived from several different plant ZFPs (Jamieson and Li, 2002). This unique zinc finger backbone had overall sequence similarities to human SP1, a natural ZFP (Cook et al., 1999). Secondly, while the first two fingers of these ZFPs were canonical C_2H_2 fingers, in the third finger the second histidine and the two adjacent upstream amino acid residues were substituted with GlyGlyCys, making

the third finger a C_2HC type (Rebar and Jamieson, 2002). Remarkably these novel ZFPs with a plant backbone and one C_2HC finger bound to their naked DNA target sequences with higher affinities and specificities than those previously reported (Zhang et al., 2000, Liu et al., 2001) for synthetic ZFPs using a more conventional backbone (data not shown). The amino acids at position "−1" through "+6" of the α-helix of each finger for each ZFP used are shown in Fig. 3B. Gel shift analysis showed that the K_d value of these ZFPs for their naked DNA target sequence ranged from 0.0001 to 0.02 nM. As a comparison, SP1 exhibited a K_d of 0.055 nM for its target under the same gel shift conditions.

Translational fusions were made between the opaque-2 nuclear localization signal (NLS), the five ZFPs, and the activation domain of C1 to generate the plant-derived ZFP-TFs (Fig. 4A). Previous studies of C1, a transcriptional activator of genes encoding biosynthetic enzyme of the maize anthocyanin pigment pathway, demonstrated that the 100 amino acids at the carboxyl-terminal were able to function as a transcriptional activator in maize, yeast and *Arabidopsis* (Guyer et al., 1998; Goff et al., 1991). A shortened C1 activation domain (60 carboxyl-terminal amino acids) was used as the ED in this study as it was found to give superior reporter gene activation in *Arabidopsis* leaf protoplast-based transient assays (data not shown).

3.3. Transcriptional activation of the endogenous Arabidopsis GMT in leaf protoplasts

Plasmids containing each of the five ZFP-TFs (A-E) cloned under the control of the constitutive 35S promoter (Fig. 4A) were transiently transfected into *Arabidopsis* leaf protoplasts and the effect on GMT mRNA levels measured by quantitative real-time PCR analysis. Four (A, B, C and E) of the 5 ZFP-TFs tested increased GMT gene expression greater than two-fold, with B, the most effective activator, giving a 5-fold increase (Fig. 4B). A dosage response experiment for B verified that the level of activation was positively correlated to the amount of DNA used (data not shown). Consistent with earlier studies showing that DNase I hypersensitivity is an indication of accessibility (Utley et al., 1997, Liu et al., 2001), the two ZFP-TFs (A and B) targeted to DNase I hypersensitive sites were found to be effective activators. Interestingly, one of the two ZFP-TFs (D and E) targeted to sites outside of the DNase I hypersensitive sites were also effective activators of the GMT gene in leaf protoplasts. These results are not entirely unforeseen as it has been previously shown that DNase I hypersensitive mapping is not sufficiently precise to reveal small stretches of accessible DNA (Zhang et al., 2000).

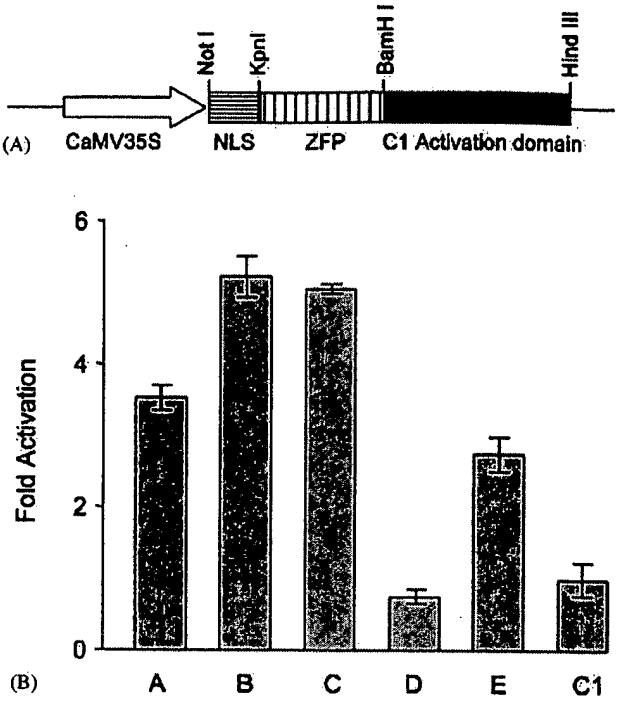


Fig. 4. Transcriptional activation of the endogenous *Arabidopsis* GMT gene by ZFP-TFs in leaf protoplasts. (A) Schematic representation of the protoplast ZFP-TF expression cassette. The cauliflower mosaic virus 35S (CaMV35S) constitutive promoter was used to drive the expression of the ZFP-TF fusion protein (maize opaque-2 nuclear localization signal (NLS), plant-derived ZFP DNA binding domain, and maize C1 activation domain). (B) Differential activation of the GMT gene by ZFP-TFs (A–E) targeted to different sites on the GMT locus. Plasmids encoding ZFP-TFs were transformed into *Arabidopsis* leaf protoplasts. Taqman quantitative RT-PCR analysis was performed on RNA extracted from protoplasts. GMT mRNA level was normalized against GAPDH mRNA and the transformation efficiency. The normalized GMT mRNA level was compared to the control vector containing only the CaMV35S driven C1 activation domain.

3.4. Phenotypic analysis of transgenic plants

The five ZFP-TFs were put under the control of the napin embryo-specific promoter and stably transformed into *Arabidopsis*. Tocopherol compositional analysis via HPLC from transgenic lines showed that segregating T2 seed contained as much as 19.8% α-tocopherol compared to an average of 1% α-tocopherol in control seed (Table 1) while total tocopherol content was unchanged (data not shown). The relative in planta efficacy of the ZFP-TFs to increase seed α-tocopherol percentage was B > A > E > D > C. There was a lot of variability in the seed α-tocopherol percentage from different lines expressing the same ZFP-TFs. Analysis of T3 seed from transgenic lines expressing either ZFP-TF A or B showed that the elevated α-tocopherol phenotype was heritable, with 26.9% being the highest α-tocopherol level recorded in T3 seed (Fig. 5). Although levels of α-

tocopherol were significantly elevated in several lines, the amount was less than that seen when the GMT cDNA itself was expressed under the control of a seed-specific promoter (Shintani et al., 1998). Significantly, the two ZFP-TFs that were targeted to DNase I hypersensitive sites (A and B) gave the highest increase in seed α-tocopherol percentage. C and E were effective activators in leaf but not in seed suggesting that these sites may not be accessible in the embryo. Hypersensitivity mapping is best performed in the target tissue. We chose to use leaf tissue in this study as we determined that it was impractical to collect a sufficient quantity of Arabidopsis embryo target tissue. Given that the GMT gene is normally poorly expressed in seed relative to the leaf it may therefore have a less accessible chromatin configuration in seed. Although it is apparent that the effectiveness of a ZFP-TF in one tissue does not guarantee its effectiveness in another, the finding that the two ZFP-TFs targeted to the major hypersensitive sites were effective in both leaf and seed tissue suggests that it is feasible to use DNaseI hypersensitivity mapping of a given gene in one tissue to predict accessible sites of that gene in another more scarce tissue type.

Table 1
Alpha-tocopherol percentage in mature T2 seed and frequency of zinc finger protein transcription factor (ZFP-TF) transgene expression in developing siliques from T1 transgenic *Arabidopsis* plants

Construct	Seed α-tocopherol (%)					Transgene expression	
	N	Mean	SEM	Min	Max	No. expressing/ no. assayed	%
Control[a]	30	1.0	0.03	0.5	1.5	0/17	0
A	18	4.5	0.68	1.3	8.8	7/13	54
B	13	6.0	1.59	1.0	19.8	4/7	57
C	35	1.6	0.03	1.1	2.1	16/16	100
D	12	2.3	0.12	1.8	3.2	12/12	100
E	22	2.9	0.23	1.3	5.5	14/17	82

[a] Controls were transformed with the parent binary vector (lacking a ZFP-TF insert).

3.5. Expression analysis of transgenic plants

TaqMan quantitative RT-PCR analysis on developing siliques containing segregating T2 seed showed good concordance between the presence of the ZFP-TF transgene and an elevated level of seed α-tocopherol, however not all transgene expressors had elevated seed α-tocopherol (Table 1). The endogenous napin mRNA level was high in all samples, confirming that the developmental stage being assayed corresponded to the time when the napin-driven transgene was expected to be expressed (data not shown). Twenty plants transformed with the two most effective ZFP-TFs in planta (A and B) and nine plants transformed with a control vector (binary transformation vector lacking a ZFP-TF insert) were selected for detailed expression analysis of C1-ED and endogenous GMT levels. Within these 20 samples there was concordance between the presence of transgene expression and elevated α-tocopherol percentage in the seed, but no correlation between relative expression level of the ZFP-TFs transgene in developing siliques and percentage α-tocopherol in mature seed of the expressors (Fig. 6). This lack of correlation may be explained by the observation that only a few copies of the ZFP-TFs would be required to completely saturate

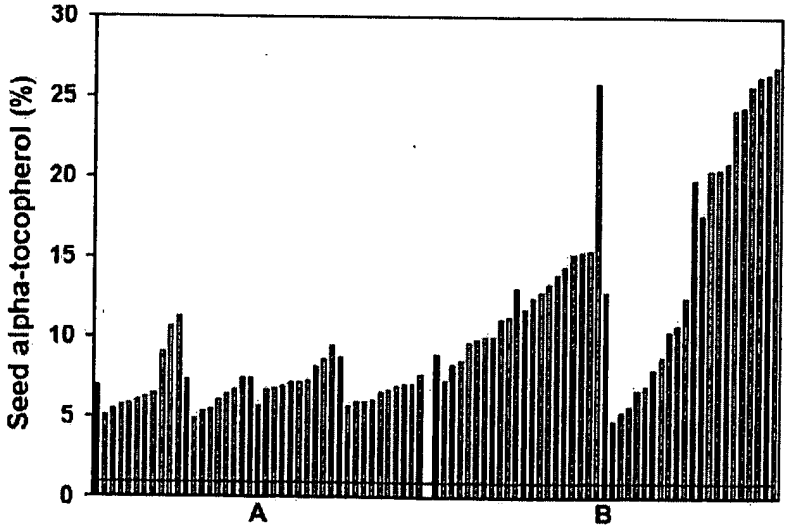


Fig. 5. α-tocopherol percentage in T2 and T3 seed from selected transgenic lines. α-tocopherol percentage in seed from individual T1 (black bar) and several T2 (gray bars) *Arabidopsis* plants derived from four independent transformation events with one of two (A or B) ZFP-TFs targeted to different recognition sites on the endogenous GMT gene. Control value of 1.0 (black horizontal line; SEM 0.03) represents the average α-tocopherol percentage measured in control seed samples from 30 plants transformed with the parent binary vector (lacking a ZFP-TF insert).

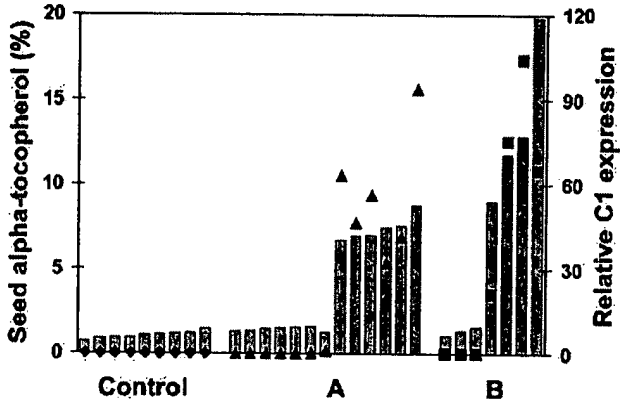


Fig. 6. α-tocopherol percentage and relative expression of ZFP-TF transgenes in developing siliques from T1 transgenic *Arabidopsis* plants. α-tocopherol percentage in T2 seed (bars) and relative expression levels of transgenic ZFP-TFs in developing siliques containing T2 segregating seed from individual transgenic plants transformed with a binary vector containing no ZFP-TF (diamond), ZFP-TF A (triangle), or ZFP-TF B (square) under the control of the napin embryo-specific promoter. Transgene expression data are normalized with 18S RNA and displayed as fold induction relative to the expression level of the weakest C1 expressor (arbitrarily set to a value of 1).

the specific binding sites, based on the size of the nucleus and the calculated in vitro dissociation constants of the ZFPs (Fig. 4). This calculation is complicated by the fact that the ZFPs may recognize other non-target sites in the nucleus. This would differentially increase the minimally effective concentration for GMT activation for each of the ZFP-TFs examined here.

There was no detectable increase in the quantity of endogenous GMT transcripts in developing siliques from these transgenic plants (data not shown). One likely explanation is that the TaqMan analysis utilized whole siliques due to the difficulty of isolating embryos from developing siliques. GMT is expressed in green, photosynthetic tissue and the high background of GMT expression in the silique walls may have made it difficult to pick up the transcriptional activation of GMT expression in transgenic embryos. It may also be that napin promoter expression of the ZFP-TFs caused an alteration in the temporal presence of endogenous GMT transcript that was not apparent in our analysis. Further expression analysis on isolated embryos from different time points in seed development would be required to assess the timing and magnitude of the transcriptional effect.

The 9 bp ZFP target sequences we chose were not expected to be unique within the *Arabidopsis* genome, and would be predicted to occur an average of every 2.6×10^5 bp. A search of the 1000 bp region upstream of all of the predicted *Arabidopsis* ORFs at The *Arabidopsis* Information Resource (TAIR) website (Huala et al., 2001) (www.arabidopsis.org/tools/) found the binding sequence of A upstream of 127 ORFs, B upstream of 96 ORFs and E upstream of 694 ORFs. Although chromatin infrastructure would likely prevent the binding of the ZFP-TFs to most of these sites, it is unlikely that all sites would be inaccessible. We had some difficulty obtaining expressing transgenic lines in three (A, B and E) of the five ZFP-TFs examined suggesting pleiotropic effects were occurring with these constructs. Using ZFP-TFs with more fingers targeting a longer DNA binding site would be one way to ensure the specificity of activation (Tan et al., 2003) and ameliorate some of these apparent pleiotropic effects.

4. Conclusion

In this study we were able to elevate the vitamin E content of transgenic *Arabidopsis* seed by expressing ZFP-TFs designed to activate the endogenous GMT gene. Our experimental approach incorporated information regarding the chromatin structure of the endogenous GMT locus and utilized plant-derived DNA sequences for the design of synthetic ZFP-TFs. Five ZFPs were designed to 9 bp DNA sequences in the promoter or coding region of the GMT gene and all were found to bind with strong affinity to their naked DNA target. ZFPs were fused to the maize C1 activation domain and four of these synthetic ZFP-TFs were found to upregulate the expression of the endogenous GMT gene in a leaf protoplast assay system. It was further shown that these ZFP-TFs were able to alter seed tocopherol composition when expressed in transgenic plants. The best line demonstrated a dramatic and heritable 20+ fold increase in seed α-tocopherol percentage. Significantly, the two ZFP-TFs that were targeted to the major DNase I hypersensitive sites in the GMT promoter gave the highest increase in seed α-tocopherol percentage. These results demonstrate that designer ZFP-TFs can be used to target endogenous gene expression and increase the amount of product derived from a specific step in a native biochemical pathway. ZFP-TF technology provides agricultural biotechnologists a powerful new method for modifying endogenous plant gene expression to achieve desired phenotypic alterations.

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